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O efeito de xenobióticos em biomarcadores de *Porcellionides pruinosus*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Susana Loureiro, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar, Departamento de Biologia da Universidade de Aveiro e co-orientação do Professor Doutor Amadeu Soares, Professor catedrático do Departamento de Biologia da Universidade de Aveiro.

Este trabalho é dedicado a toda a minha família mais próxima, a minha mãe Maria de Lurdes de Carvalho Ferreira, o meu irmão Ricardo Jorge de Carvalho Ferreira, á minha avó Emília Cabral de Carvalho e á memória do meu avô Álvaro Marques Ferreira, sem os quais não chegaria nem perto da pessoa que sou agora.

o júri

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palavras-chave Biomarcadores, isopodes, reservas energeticas, zinco e diazinon

resumo

A enorme quantidade de contaminantes quer produzidos pelo Homem (p.e. PHAs, pesticidas, organofosfatos, organocloretos, PBDEs), quer presentes na natureza (p.e. metais) tem um efeito significativamente adverso nos organimos encontrados no meio ambiente. Nos últimos anos, vários biomarcadores têm sido usados na avaliação do efeito de contaminantes no meio ambiente, contudo quase nenhuma informação se centrou no uso desta ferramenta em organismos detritívoros como os isópodes.

As reservas energéticas (açucares, lipidos e proteínas) são essenciais para os requisitos de manutenção, crescimento e reprodução de qualquer organismo. As reservas energéticas juntamente com os parâmetros actividade dos sistema de transporte de electrões (STE) e alocação de energia celular (AEC) podem fornecer-nos informação sobre a condição dos organismos quando afectados por contaminantes.

Organismos de solo, com o isópode *Porcellionides pruinosus*, são essenciais para o bom funcionamento dos ecossistemas. Como macrodecompositores, alimentando-se principalmente de matéria vegetal morta, têm um papel importante na cadeia detritívora, através da fragmentação do húmus e pela estimulação e/ou ingestão de fungos e bactérias. São, por isso, organismos importantes na reciclagem de nutrientes. O uso destas espécies chave, juntamente com biomarcadores e conteúdos energéticos poderá ser uma boa ferramenta na avaliação de risco ambiental (ARA).

Neste estudo foi avaliado o efeito dos contaminantes zinco e diazinão fornecido por exposição a comida contaminada no isópode *Porcellionides pruinosus* (Brandt 1833). O estudo baseou-se principalmente no padrão observado nos biomarcadores e nas reservas energéticas para dois tempos de exposição e duas concentrações, já posteriormente descritas como provocando nenhum efeito ou pouco efeito.(5.5 mg zinco/g folha seca, 9.5 mg zinco/g folha seca e de 17.5 µg diazinão/g folha seca, 175 µg diazinão/g folha seca, respectivamente). Para os biomarcadores o tempo de exposição foi de 96h e 7 dias e para as reservas energéticas foi de 7 dias e 14 dias.

Os biomarcadores testados foram a acetilcolinesterase (ACHE), lactato desidrogenase (LDH), glutationa *S*-transferase (GST), glutationa peroxidase (GPx), catalase (CAT) e peroxidação lipídica (LPO). As reservas e conteúdos energéticos medidos foram açúcares, lipidos, proteínas, STE e AEC.

Para a exposição a zinco, os biomarcadores GST, CAT e LPO parecem corresponder aos resultados obtidos em outros trabalhos. As reservas energéticas afectadas com uma diminuição significativa foram os açúcares, apresentando também um decréscimo nos valores de ETS e CEA.

A exposição a diazinão apresentou diferenças significativas apenas para a ACHE, não apresentado nenhum dos outros biomarcadores alterações de padrões na sua actividade, excepto a GPx para um tempo de exposição de 14 dias. Os lipidos e açúcares foram afectados pela exposição a diazinão e verificou-se também uma diminuição na AEC.

keywords Biomarkers, isopods, energy reserves,zinc and diazinon

abstract

The enormous amount of contaminants produced by man (i.e. PAHs, pesticides, organophosphates, organochlorides, PBDEs), or that can be found in nature (i.e. metals) has a significant adverse effect on organisms present in the environment. In recent years the biomarkers have been used to evaluate the effects of these contaminants in the environment, but few data has been focused on this assessment tool using detritivorous key-organisms like isopods.

Energy reserves (carbohydrates, lipids and proteins) are important for the maintenance, growth and reproduction requirements of any organism. Energy reserves along with the electron transport system activity (ETS) and with the cellular energy allocation (CEA) can give us information about the organisms "status" when affected by the contaminants.

Soil key-dwelling organisms like the isopod *Porcellionides pruinosus*, are essential to the ecosystems' functions. As macrodecomposers (feeding mainly on decaying plant material) they play an important role in the detritus food chain, through litter fragmentation and stimulating and/or ingesting fungi and bacteria that are important in the cycling of nutrients. The use of these key species along with biomarkers and energy budgets can be a good environmental risk assessment (ERA) endpoint

In this work the effects of two environmental contaminants (zinc and diazinon) through food exposure were studied using the isopod *Porcellionides pruinosus* (Brandt 1833). The study is mainly focused on the effect patterns observed for the biomarkers and energy reserves for two time exposure and two concentrations presented as NOEC and LOEC on previous studies(5.5 µg zinc/g dry leaf, 9.5 µg zinc/g dry leaf and 17.5µg diazinon/g dry leaf, 175µg diazinon/g dry leaf respectively). For biomarkers the exposure time was 96h and 7-days, as for the energy reserves was 7-days and 14-days.

The biomarkers tested were acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione *S*-transferase (GST), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO). The energy reserves and budget tested were: carbohydrates, lipids, proteins, electrons transport system activity (ETS) and cellular energy allocation (CEA).

For zinc exposure the biomarkers GST, CAT and LPO seem to correlate with results obtained for other works. The energy reserves affected were the cabohydrates with significant decrease in their content along with a decrease in both ETS and CEA.

The diazinon exposure showed only significative results for AChE, with no changes in all the other biomarkers activity, except the GPx for a 14-day exposure. The energy reserves were affect by a decrease in the carbohydrate and lipid content along with a decrease in the CEA.

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CHAPTER I

Introduction, thesis structure and objectives

Introduction

In the current days the concern on the environment quality and health is being taken in great consideration. Every chemical compound that has a potential release into the environment or be in contact with humans is being studied and programs like the new European legislation REACH are being created to regulate the use of such compounds. The major part of the tests used to assess toxicological effects are based on phenotypical observations (e.g. mortality, growth, reproduction, food consumption) and although they give information on the organism status, they do not always transmit the real condition of the organism and possible long term effects. The use of biomarkers will allow us to test subindividual and non-observable parameters like enzyme activity that can be also used as early warning tools because they can provide information on the organism health before a symptom can be observed.

Biomarkers

The definition for biomarkers or biological indicators not only varies from author to author, but also to the scientific area where it is applied. For example Mendelsohn et al. (1998) describe biomarkers has observable properties of an organism that can be used to identify the organism's presence, as in microbiology or forensic pathology; to estimate the organism's prior exposure, as in risk assessment; to identify changes or effects occurring in the organism, as in toxicology and diagnostic medicine; or to assess the underlying susceptibility of the organism, as in genetics and pharmacology (Mendelsohn et al. 1998).

In a general way definition of biomarker for ERA can be given as any biological response to an environmental contaminant at a sub-individual level, measuring biochemical, molecular, genetic, immunologic, physiologic signals or even organism products (e.g. urine, faeces, hair, feathers, etc.) of events in biologic systems (vanGestel & vanBrummelen 1996). These events indicate a deviation from the normal status and most of the times cannot be detected in the intact organism. The definition of biomarkers also come associated to two predominant features: (1) their sensitivity and quick response may give early alarms with regard to toxicant impacts on organisms,

well before ecological disturbances can be observed, (2) they may give a more direct and accurate relationship between toxicant exposure and biological response (Morgan et al. 1999).

Biomarkers have been classified in three categories: biomarkers of exposure, effect or susceptibility (WHO, 2001):

- Biomarker of exposure: the result of an interaction between a contaminant and a target molecule or cell that is measured in a compartment in an organism.
- Biomarker of effect: an alteration in an organism that, depending on the magnitude, can be associated with a possible health condition or disease.
- Biomarker of susceptibility: a specific response of an organism when exposed to a specific toxic (NRC 2006).

Although these separation can be made is also important to underline that with the increase of scientific knowledge the delineation between these classifications may change (NRC 1987).

Several biomarkers have been evaluated in key-species used in ecotoxicological tests. Their methodology is usually based in a basic principal for the respective target molecule/enzyme and then adapted according to the organism used.

The following part of this section will include an overall description of several biomarkers usually used in ecotoxicological approaches.

Acetylcholinesterase (AChE)

Acetylcholinesterase is an enzyme responsible for the degradation of the neurotransmitter acetylcholine, producing choline and an acetate group (Fig. 1) and can be found on the anterior part of nerve terminals (Purves et al. 2008).

Acetylcholinesterase (AChE) is a biomarker representative of direct enzyme inhibition, that is linked with the mechanism of toxic action where a irreversible or reversible binding to the esteratic site and potentiation of cholinergic effects occurs.

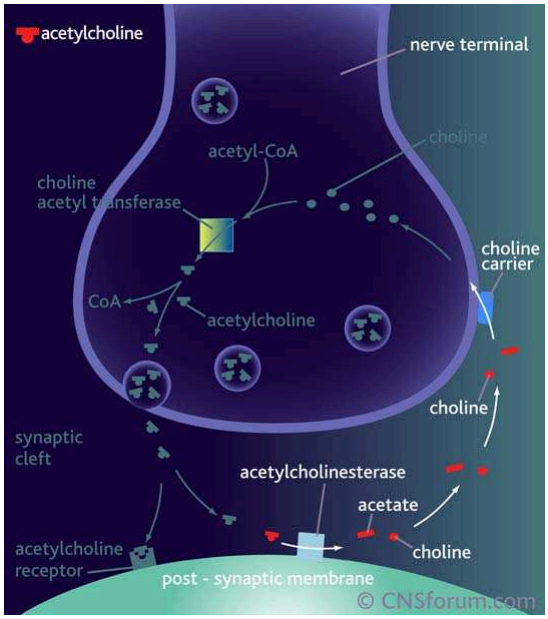

Fig. 1 Scheme for the acetylcholinesterase mechanism of action (adapted from www.chm.bris.ac.uk)

The quantification of AChE activity has already been use for field and laboratory studies to assess exposure for antipsychotics (Seibt et al. 2009), organophosphates and carbamate insecticides (Drobne et al. 2008, Elumalai et al. 2002, Jadhav & Rajini , Ray et al. 2009, Ribeiro et al. 1999, Stanek et al. 2006, Xuereb et al.), herbicides (Moraes et al.), antibiotics (Tu et al. 2009) or metals (Calisi et al. 2009, Elumalai et al. 2002, Gill et al. 1990). For invertebrates compounds responsible for the inhibition of AChE are lethal and have a profound population impact (Huggett et al. 1992).

Although cholinesterase (ChE) activity is often used as a biomarker for effects of anticholinesterase pesticides, its use requires a characterization and activity range measurements (Bocquene et al. 1990, Garcia et al. 2000).

Cholinesterases are traditionally divided into two classes, AChE (EC 3.1.1.7) and butyrylcholinesterase or pseudocholinesterase (BChE, EC 3.1.1.8) (Monteiro et al. 2005). These two classes of enzymes can be distinguished based on the substrate specificity and their susceptibility to selective inhibitors (Kozlovskaya et al. 1993). Studies also show that more than one ChE may be present within the same organism (Kozlovskaya et al. 1993, Sturm et al. 1999, Sturm et al. 2000).

Lactate dehydrogenase (LDH)

Lactate dehydrogenase is an enzyme of the intermediary metabolism group (Fig. 2), and its responsible for the reduction of pyruvate to lactate, being also important in the redox maintenance (Huggett et al. 1992). This enzyme activity is considerable influenced by factors like temperature, season, diet, sex, and reproduction condition. Increases in LDH activity has been reported for invertebrates exposed to xenobiotics (e.g. (Diamantino et al. 2001, Ribeiro et al. 1999)

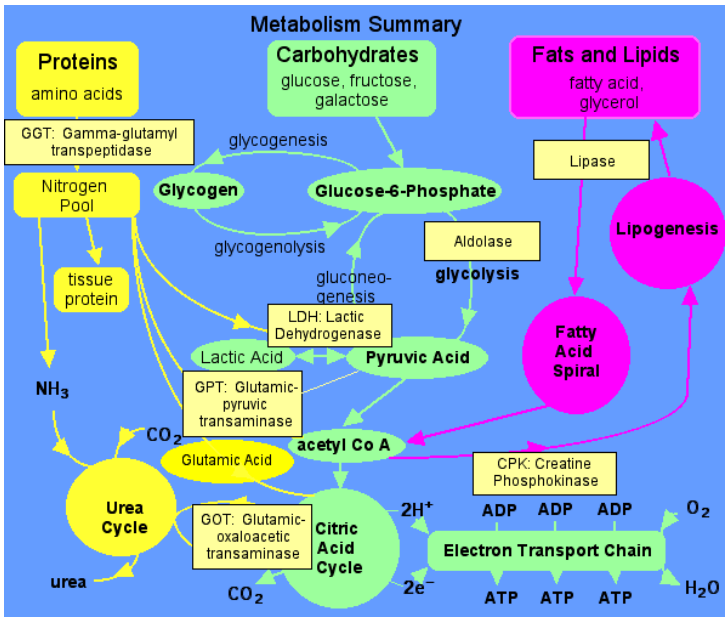

Fig. 2 Scheme for the lactate dehydrogenase role and relationship to the overall metabolic processes (adapated from www.elmhurst.edu)

Catalase (CAT)

Catalases (CAT) are hematin-containing enzymes that facilitate the removal of H_2O_2 from the organism. The main activity of CAT is associated with the peroxisomes or microbodies that function on the fatty acid metabolism (Huggett et al. 1992). Catalase activity appears to be connected along with the glutathione peroxidase (GPx) activity to combat the oxidant stress exposure (Diesseroth & Dounce 1970). The catalase function can be described by the following:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Glutathione Peroxidase (GPx)

Glutathione peroxidase similary to CAT has as main target the molecule of H_2O_2 and employs reduced glutathione (GSH) as cofactor, as showed by the following:

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

Glutathione peroxidase can also catalyse the reduction of organic hydroperoxides to the corresponding alcohols (i.e. ROOH → ROH), considered an important mechanism for altering lipid peroxidizing chain reactions (Huggett et al. 1992). Increases in GPx activity has been already reported for several vertebrates or invertebrates exposed to xenobiotics (Howcroft et al. 2009, Labrot et al. 1996, Marie et al. 2006).

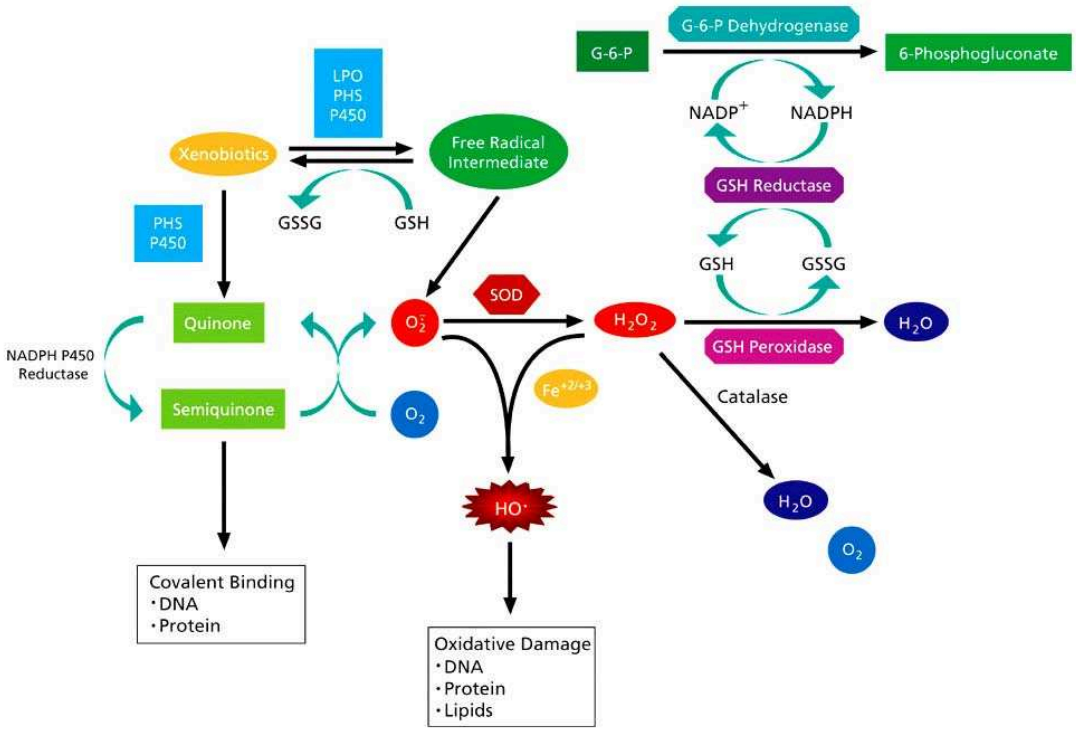

Fig. 3 Scheme for the oxidative stress cycle (from Sigmaaldrich.com)

Glutathione S-Transferase (GST)

The glutathione *S*-transferase (GST) represents a family of enzymes acting as catalysts for the conjugation of various electrophilic compounds with the tripeptide glutathione (Armstrong 1987, Gulick & Fahl 1995). In their role for detoxification, they are responsible for the increase of available lipophilic toxicants to phase I enzymes, serving as carrier proteins or by covalently binding to electrophilic compounds themselves which reduces the likelihood of these compounds to bind to other macromolecules such as DNA (Schelin et al. 1983). In mammals various classical inducers of drug metabolism (e.g. PAH, PB and PCB) have been identified as GST activity inducers (Huggett et al. 1992).

Lipid Peroxidation (LPO)

Oxidative stress has a great impact on the oxidation of polyunsaturated fatty acids (Huggett et al. 1992). Lipid peroxidation involves a long process that, at its end, can react with transition metal complexes (including the phase I detoxification enzyme – cytochrome P450 (Huggett et al. 1992)). Several studies have demonstrated enhancements of lipid peroxidation in several tissues due to diverse xenobiotics or even as consequence of cellular damage; but alone LPO is insufficient to indicate any base of toxicity from compounds that causes oxidative stress. So, it should always be accomplish by other oxidative stress biomarker such as the superoxide dismutase (SOD).

Energy Reserves

Xenobiotics and stressors can induce changes on the concentration of stored energy reserves which are important for the maintenance, growth and reproduction requirements of any organism. These energy reserves are normally stored has glycogen or lipids and are used whenever necessary to one of the above requirements. Under severe conditions caused by xenobiotics or stress beside the use of glycogen and lipids, proteins can also be used although they are not store for these propose (Huggett et al. 1992).

Glycogen forms an energy reserve that can be quickly mobilized to meet a sudden need for glucose. It increases and decreases in glycogenolysis and its storage and mobilization is restricted to certain tissues (Huggett et al. 1992). In vertebrates glycogen content is affected by acute and chronic exposures to metals and organic compounds (Bhagyalakshmi et al. 1983, Graney & Giesy 1986, Thomas et al. 1981) and its depletion has been attributed to the increased energy demand associated with chemical-induced stress (Huggett et al. 1992). The measurement of glycogen represents a useful measurement of the relative energy status of an organism in time and can be predictive of higher level effects (Huggett et al. 1992).

Lipids are an essential and ready energy source that varies as primary or secondary source to be wasted within species and season. Such as glycogen, the distribution of lipids varies within tissues and is influenced by factors like temperature or reproductive conditions. In most cases where stress in chemically induced, a decrease on lipid contents is observed but often classified as not significant (Huggett et al. 1992). As glycogen, lipids represent a useful measurement of energy reserves although are normally considered a secondary energy source after glycogen. So they usually used for long-time exposure tests (Huggett et al. 1992).

Proteins represent a large percentage of an organism's body, since they are responsible for the body structure and are influenced by a variety of environmental factors. Under severe conditions invertebrates can mobilize proteins as an energy source by the oxidation of amino acids, being used normally after glycogen and/or lipids are used (Bayne 1973, Giles 1984). The measurement of proteins as energy source has normally little utility, unless the stress induced by the experimental procedures is extremely high (Huggett et al. 1992).

Cellular Energy Allocation (CEA)

The whole-body caloric content of organisms can be calculated by converting the energy reserves to caloric equivalents. A study published by De Coen et al. (1995) presented the technique Cellular Energy Allocation (CEA) assay, to evaluate the effects of toxic stress on the metabolic balance of test organisms. This assay is based on the

energy reserves available (E_a) and energy consumption (E_c) quantified, and its integration into a general stress index. Energy consumption (E_c) is estimated by measuring the electron transport activity (ETS) at a mitochondrial level, and the energy available (E_a) by measuring the total lipid, protein and carbohydrate content for the tested organism. This data in them divided by the time exposure period (t) using the following formula:

$$CEA\,(J/mg\ org) = \frac{(\int_0^t E_a.dt - \int_0^t E_c.dt)}{t}$$

The different energy reserve fractions (E_a) for the individual organisms are transformed into energetic equivalents using the energy of combustion (Gnaiger 1983): 17.5 J/mg glycogen, 24 J/mg protein and 39.5 J/mg lipid. The cellular respiration rate (E_c) is determined, using the ETS data, based on the theoretical stoichiometrical relationship that for each 2 µmol of formazan formed, 1 µmol of O_2 was consumed in the ETS system. The quantity of oxygen consumed was then transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 480 kJ/mol O_2 (Gnaiger 1983).

Isopods and exposure routes in Ecotoxicology

Terrestrial isopods (woodlice) are successful invaders of the terrestrial habitats among crustaceans. They are more related to crabs or lobsters then to terrestrial arthropods such as insects or spiders (Lokke & vanGestel 1998).

Similar to all Arthropoda, woodlice have a segmented body, with a rigid exoskeleton and jointed limbs. They possess three groups of segments, the head, followed by the thorax or pereion, and finally the abdomen or pleon (Fig. 4). They also present a pair of eyes and antennae (Sutton 1980).

Also for their internal structures, woodlice have typical arthropod's structures, with a pair of ganglia above the oesophagus receiving nerve tracks from the eyes and antennae, ganglia connected by commissures to the suboesophageol ganglia. A ventral nerve cord

with a more or less fused pair of ganglia in each pereion segment and a large fused pleon ganglion runs through the length of the body, starting in the suboesophageol ganglia as showed in Fig. 5 (Sutton 1980). The digestive system is basically composed by a strait gut that passes from the oesophagus into the proventriculus, the structure responsible for grinding the food and filtering the juice and small particles, passing them through the hepatopancreas (Sutton 1980). The reproductive system is very simple consisting in a pair of trilobed testes with a duct to the genital papilla in males. In females, a pair of ovaries opens thought oviducts into the brood pouch. It is known that female isopods can store sperm and it is presumable that this storage in made on the walls of the oviduct (Sutton 1980).

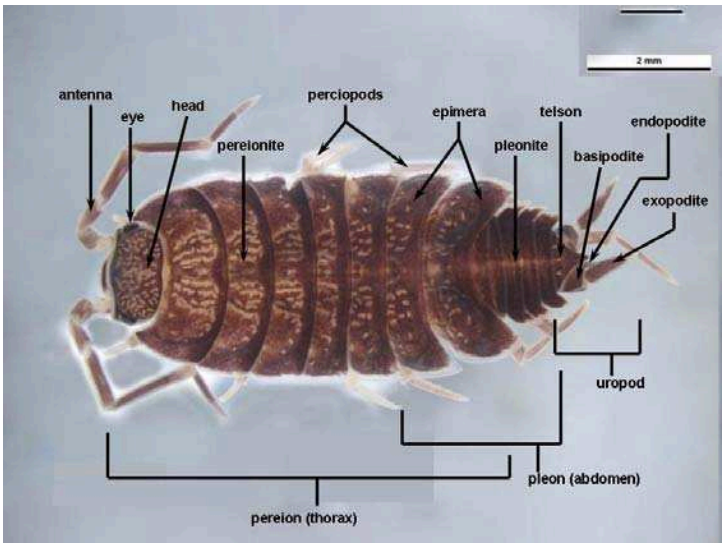

Fig. 4 Dorsal view of Porcellionides pruinosus (adapted from Callan & Graham (2006))

Woodlice appear in almost all types of ecosystems and habitats ranging from seashores to dry or even desert lands (Lokke & vanGestel 1998). Seasonal climatic changes can cause migrations to avoid desiccation or even activity, and they are considered nocturnal organisms. This specie nocturnal activity also determines as its most important predators: beetles, spiders, centipedes, toads, shrews and birds (Lokke & vanGestel 1998).

As cryptozoic animals, they present an aggregating behavioural response, hiding during the day under stones, bark or even thick leaf litter in places with high humidity (Lokke & vanGestel 1998). Isopods' reproduction strategy is iteroparous and animals have several broods within a year

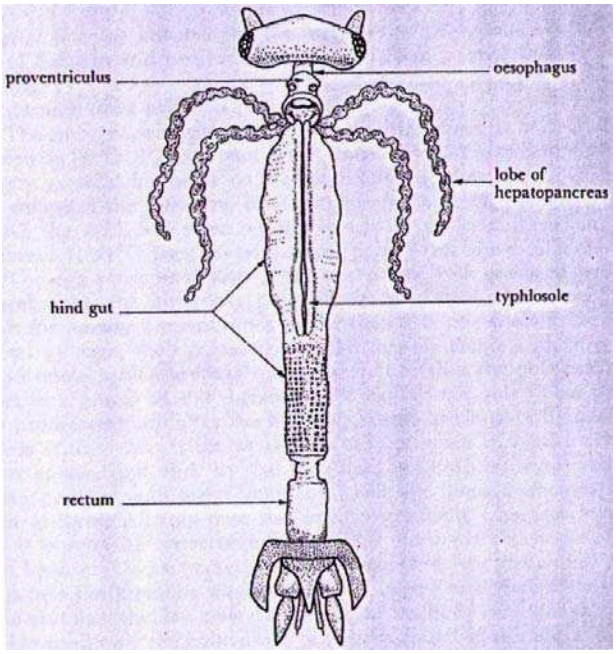

Fig. 5 Scheme for the dorsal view of the dissected digestive system (from Sutton 1980)

They are macrodecomposers, feeding mainly on decaying plant material, and play an important role in the detritus food chain, through litter fragmentation and stimulating and/or ingesting fungi and bacteria that are important in the cycling of nutrients (Loureiro et al. 2006).

Although isopods ingest a large amount of food, their assimilation efficiency is rather low depending also in the type of food quality. A study made by Loureiro et al (2006) based in the different feed performance of *P. pruinosus*, when fed with alder (*Alnus glutinosa*), oak (*Quercus robur*), eucalyptus leaves (*Eucalyptus globulus*) and pine needles (*Pinus sp.*), showed an assimilation efficiency of 87%, 68%, 45% and 41%, respectively.

Contaminated ecosystems induce deleterious effects on soil-dwelling organisms. The exposure routes to these organisms include the uptake via soil, but also by the litter-layer where chemical compunds like metals can be accumulated (Martin et al. 1982). So detritivorous organisms like isopods face high metal exposures, since their food source is the litter-layer. As an example, a study done by Vijver et al. (2006) showed that zinc uptake rate constants from food were slightly lower than from soil and concluded that

the relative importance of the uptake sources depends mainly on the partitioning of metals between soil and food.

Xenobiotics that are up taken through food enter orally to the digestive tract and go directly from the gut fluid or via the typhsole channels to the hepatopancreas (Hames & Hopkin 1991). Xenobiotics may also diffuse into the haemolymph, to be partly osmoregulated via the maxillary glands (Donker et al. 1996). Another possible route is via the pleopods structures that absorb water from the environment by capillary action (Sutton 1980), and leads xenobiotics to circulate in the haemolymph through the entire body until a target organ is reached.

Objectives

The main goal of this study was to develop and carry out a battery of molecular biomarkers in the terrestrial isopod Porcellionides pruinosus. With this tool, basal levels for biomarkers were determined and biomarker patterns when exposed to the heavy metal zinc and the pesticide diazinon assessed.
To accomplish the main objective, the work was divided into the following steps:

1. Determination of the best homogenization method to use;
2. The basal activity levels of biomarkers: acetylcholinesterase (AChE), Lactate Dehydrogenase (LDH), and the oxidative stress biomarkers Lipid Peroxidation (LPO), Gthutathione *S*-Transferase (GST), Gluthathione Peroxidase (GPx) and Catalase (CAT);
3. The basal activity levels of energy reserves (lipids, proteins and carbohydrates);
4. Response patterns of all biomarkers referred above and respective quantification of energy reserves along with the Cellular Energy Allocation (CEA) parameter, when isopods were exposed to chemical compounds (diazinon and zinc)
5. Correlation between all of the measured parameters.

Zinc (Zn) is one of the essential trace elements for animal nutrition, having structural, catalytic and regulatory functions in organisms (Maret 2005, Takeda 2000). It is essential for the action of over 300 enzymes and necessary in metallothioneins, thioneins, DNA replication, transcription and protein synthesis (Augustyniak et al. 2006). Zn has proved to be toxic to several terrestrial isopod species, showing

impairment on feeding rates, energy reserves and body size (Drobne & Hopkin 1995, Jones & Hopkin 1998, Loureiro et al. 2006, Shu et al.).

For the pesticide exposure, diazinon (O,O-diethyl-O-(2-isopropyl-6-methyl-pyrimidine-4-yl)phosphorothioate - Fig. 6) was chosen. It is a nonsystemic organosphosphate insecticide and acaricide developed in the early 1950s. It is used throughout the world to control public health, and is applied to control ectoparasites in veterinary medicine (Watterson 1998). Diazinon was heavily used during the 1970s and early 1980s for general-purpose gardening use and indoor pest control. Diazinon kills insects by inhibiting acetylcholinesterase, an enzyme necessary for proper nervous system function. Diazinon has a low persistence in soil, and is degraded by hydrolysis, photolysis and microbial metabolism, having a half-life in soil from 17 to 39 days (ABC 2009). This pesticide has been banned in the US since 2005 (ABC 2009)

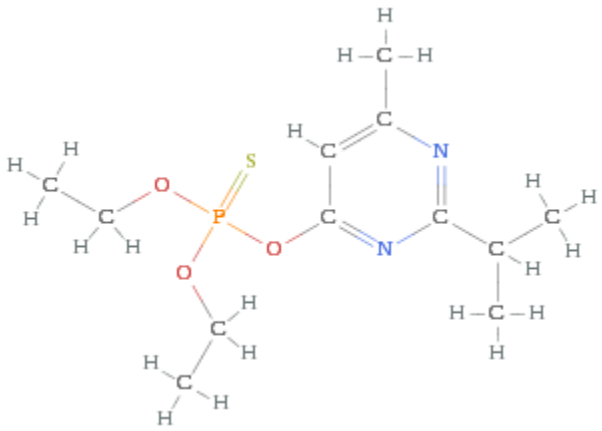

Fig. 6 Diazinon structure diagram (from wolframalpha.com)

Thesis structure

The present thesis is organized in the following chapters:

- *Chapter I* – Introduction, thesis structure and objectives.
- *Chapter II* – Basal levels of biomarkers and energy reserves in *Porcellionides pruinosus*.
- *Chapter III* – Effects of the metal zinc and the pesticide diazinon on biomarkers of *Porcellionides pruinosus*.
- *Chapter IV* – Effects of Zinc and Diazinon on the energy budget of the isopod *Porcellionides pruinosus.*
- *Chapter V* – Discussion and Conclusion.

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CHAPTER II

Basal levels of biomarkers and energy reserves in Porcellionides pruinosus

Basal levels of biomarkers and energy reserves in *Porcellionides pruinosus*

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Abstract:

In the last decades biomarkers have been widely used for the assessment of effects and/or exposure to environmental contaminants, but few or none data has been determined for isopods, detritivorous key-organisms. Along with biomarkers the quantification of the energetic reserves has also been used to evaluate organisms energetic budget. One of the most frequently used biomarker is the inhibition of cholinesterases (ChE), which is a useful indicator of organophosphate and carbamate exposure and/or effects.

In this study, the cholinesterase activity of the isopod *Porcellionides pruinosus* was characterized using three substrates (acetylthiocholine iodide, propionylthiocholine iodide, and *S*-butyrylthiocholine iodide) and three ChE inhibitors (eserine hemisulfate, BW284C51, and iso-OMPA). The sample homogenization method was also tested (homogenizator/sonicator), extracting respectively 3.34 ± 0.53 mg of protein and 2.75 ± 0.40 mg of protein (mean ± st. error).

Other biomarkers related to oxidative stress or metabolism were assessed. The basal range of biomarkers activity was AChE 113.56 ± 4.71 U/mg protein, LDH activity 3.03 ± 1.11 U/mg protein, CAT 6.11 ± 1.13 U/mg protein, GPx 2.73 ± 1.07 U/mg protein, LPO 34.57 ± 4.66 U/mg ww and GST 137.76 ± 7.13 U/mg protein (mean ± st. error). The mean carbohydrates and protein content was respectively 12290.76 ± 56.40 J/mg org and 22904.98 ± 57.46 J/mg org (mean ± st. error). The lipid content was 503.14 ± 12.74 J/mg org.

The present study underscores not only the relevance of ChE characterization before its use as a biomarker in biomonitoring studies, but also the homogenization method and basal levels for biomarker activity and energetic reserves along with its comparison to other previous works. These data will be very useful and crucial as foundation for other monitoring or ecotoxicological testing.

***Keywords*:** biomarkers, energy reserves, isopods, basal levels

1. Introduction

On a daily basis we are surrounded by xenobiotics that are causing a great impact on human health and on the environment. So it is important to screen and analyse the effects of these xenobiotics and properly assess and manage these risks, as part of an Environmental Risk Assessment (ERA) procedure. To accomplish this task is important to have new, fast and accurate tools. The development of biomarkers based on the study of biological responses of organisms to pollutants has proved to be essential biochemical tools to the implementation of programs for monitoring contaminant exposure and/or effects.

Soil is seriously affected by xenobiotics and the cleaning of contaminated soils is more complex and difficult than water and air, so with the increase of pollution, key soil-dwelling organisms like terrestrial isopods, will be put in risk for longer periods. Terrestrial isopods (woodlice) are successful invaders of terrestrial habitats, and are essential to the ecosystems' functions. As macrodecomposers (feeding mainly on decaying plant material) they play an important role in the detritus food chain, through litter fragmentation and stimulating and/or ingesting fungi and bacteria that are important in the cycling of nutrients (Loureiro et al. 2006). The use of these key species along with biomarkers and energy budgets can be a good ERA tool.

Biomarkers can be described has any biological response to an environmental chemical below-individual level, measuring biochemical, molecular, genetic, immunologic, physiologic signals or even organism products (e.g. urine, faeces, hair, feathers, etc.) of events in biologic systems (vanGestel & vanBrummelen 1996). These events indicate a departure from the normal status and most of the times cannot be detected in the intact organism. The definition of biomarkers also come associated to two predominant features: (1) their sensitivity and quick response may give early alarms with regard to toxicant impacts on organisms, before ecological disturbances can be observed, (2) they may give a more direct and accurate relationship between toxicant exposure and biological response (Morgan et al. 1999). Two main advantages of using biomarkers as a tool is that they can give linkage information not only on the quantification of the pollutant but also on the effects and mode of acting on the organisms, and can assess

early stages of "status" change below individual levels that can not become apparent in other types of tests (Kammenga et al. 2000, vanGestel & vanBrummelen 1996).

Along with biomarkers energy reserves can be a good endpoint in ERA since they are necessary for repairing mechanisms and eventually pathological effects, that result from continuous or pulse exposure to contaminants. This energy costs may also be needed to resist the toxicant by avoidance, exclusion, or removal (Ribeiro et al. 2001).

But the use of biomarkers or the quantification of energy reserves on isopods, as for other species, can not be done without the determination of the basal levels since these values are essential to evaluate the significance of biomarkers measurements in laboratory studies.

The main objective of this study was to evaluate the basal levels of several molecular biomarkers and energy reserves in the terrestrial isopod *Porcellionides pruinosus*. For that several objectives were defined: i) to characterize the cholinesterase present in this isopod; ii) to determine the best homogenization method to use: homogenizer vs sonicator; iii) determine the basal activity of the biomarkers acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione *S*-transferase (GST), catalase (CAT), lipid peroxidation (LPO), glutathione peroxidase (GPx); iv) quantify energy reserves (lipids, proteins and carbohydrates); iv) compare all data with other previous published for species from the same taxa or other key-species.

2 Materials and methods

2.1 Test Organism and Culture Procedure

The organisms used in these experiments belong to the specie *Porcellionides pruinosus* (Brandt, 1833), and were previously collected from horse manure pills and maintained for several generations in laboratory cultures. In this cultures isopods are fed *ad libidum* with alder leaves (*Alnus glutinosa*) and maintained at 25 ± 2°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water spayed and extra food is

provided. Only adult animals (15-25 mg wet weight) were used in the experiments; there was no distinction between sexes, although pregnant females were excluded.

2.2 Experimental procedure

Test organisms were collect from culture boxes, weighted and visually observed: animals with abnormalities, moulting and pregnant females were discarded. Assays were performed, using a pool of two organisms to test the biomarkers: glutathione *S*-transferase (GST), glutathione peroxidase (GPx), catalase (CAT), and lipid peroxidation (LPO). One organism was used and divided into head and body to test acetylcholinesterase (AChE) and lactate dehydrogenase (LDH), respectively.

To quantify the energy reserves (lipids, carbohydrates and proteins) one organism was used for carbohydrates and proteins and another one for lipids.

Twenty replicates were used to determine all enzymatic activities and quantify the energy reserve content. In order to optimize the methodology for these measurements two procedures were applied to our sampling animals. Ten organisms were processed using a homogenizer and the other ten were processed using a sonicator.

2.3 Cholinesterase Characterization

Cholinesterase characterization was performed by determining substrate preferences and selective inhibitor effects. A pool of twelve heads from culture organisms were homogenized using a sonicator in 6 ml of K-Phosphate buffer (0.1M, pH 7.2) and centrifuged (1 700 g, 3 min, 4ºC) for cholinesterase activity determination, which was performed with six replicates, according to the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996).

In independent experiments, acetylthiocholine iodide (AcSCh), *S*-butyrylthiocholine iodide (BuSCh), and propionylthiocholine iodide (PrSCh) within a dose range were used as substrates. Eserine hemisulfate was used as selective inhibitor of the activity of all the ChE, tetraisopropyl pyrophosphoramide (iso-OMPA) as selective inhibitor of pseudocholinesterase (PChE) and 1,5-bis(4-allyldimethyl-ammonimphenyl) pentan-3-one dibromide (BW284C51) as selective inhibitor of AChE. The enzymatic activities

were determined with AcSCh after an incubation period of 30 min at 25 ± 1ºC. For each inhibitor, 5 µl of a stock solution was incubated with 495 µl of homogenate ample extract. Inhibitor concentrations ranged from 6.25 to 200 mM (eserine and BW284C51) and from 0.25 to 8.0mM (iso-OMPA). Ultrapure water was added to controls and an additional control with ethanol was used in the experiments with iso-OMPA.

2.4 Post-mitochondrial supernatant (PMS)

Each replicate composed of two organisms were homogenized using a homogenizer or a sonicator in 1 ml K-Phosphate 0.1 M buffer, pH 7.4. From the homogenate 300 µL were separated into a microtube and 5 µL butylated hydroxytoluene (BHT) 4% in methanol were added for endogenous lipid peroxidation (LPO) determination. The remaining tissue homogenate (700 µL) was centrifuged at 10 000g for 20 min. (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was divided into four microtubes for posterior analysis of biomarkers and protein quantification. All microtubes were stored at -80ºC until analysis, for a period no longer than 1 week.

2.5 Lipid peroxidation (LPO)

The lipid peroxidation (LPO) assay was based on the method described by Bird & Draper (1984), Ohkawa et al. (1979) by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 300 µL homogenated tissue, 1 mL TCA 12% (w/v), 1 mL TBA 0.73% (w/v) and 800 µL Tris-HCl 60mM with DTPA 0.1 mM. The reaction mixture was then incubated at 100ºC in a water bath for 1h. After this, tubes were centrifuged for 5 min. at 11 500 rpm (25ºC). Samples were kept away from light, at 25ºC and immediately read at 535 nm. The enzyme activity is expressed as unit (U) per mg of wet weight. A U is a nmol TBARS hydrolyzed per minute, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

2.6 Glutathione S-Transferase (GST)

Glutathione *S*-Transferase (GST) activity was determined based on the method described by Habig et al. (1974) and adapted to microplate (Diamantino et al. 2001). We mixed 100 µL of PMS in 200 µL of a reaction solution. The reaction solution was a mixture of 4,950 ml K-Phosphate 0.1 M (pH 6.5) with 900 µL GSH 10mM, and 150 µL 1-chloro-2,4- dinitrobenzene (CDNB) 10mM. It was measured at 340 nm. The enzyme

activity is expressed as unit (U) per mg of protein. A U is a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6 $x10^{-3}$ M^{-1} cm^{-1}.

2.7 Glutathione Peroxidase (GPx)

Glutathione Peroxidase (GPx) activity was determined based on the method described by Mohandas et al. (1984). We mixed 50 µl PMS with 840 µl K-Phosphate 0.05 M (pH 7.0) with EDTA 1 mM, Sodium azide 1 mM and GR (7.5 mL from stock with 1 U/mL), and 50 µL GSH 4 mM, by measuring the decrease in NADPH (50 µl, 0.8 mM) at 340 nm and using H_2O_2 (10 µl, 0.5 mM) as substrate (Mohandas et al. 1984). The enzyme activity is expressed as unit (U) per mg of protein. A U is a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.22$x10^{-3}$ M^{-1} cm^{-1}.

2.8 Catalase (CAT)

Catalase (CAT) activity was determined based on the method described by (Clairborne (1985). We mixed 50 µl of PMS with 500 µL H_2O_2 0.030 M, and 950 µL K-Phosphate 0.05 M (pH 7.0) and measured the decomposition of the substrate (H_2O_2) at 240 nm. The enzyme activity is expressed as unit (U) per mg of protein. A U is a µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

2.9 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) activity was determined at 340nm by the method of Vassault (1983) adapted to microplate by Diamantino et al. (2001). Whole body was homogenized using a homogenizer or a sonicator in 500 µl of TRIS/NACl buffer (0.1M, pH 7.2), the supernatants obtained after centrifugation of the homogenates (4 ºC, 1 700 g, 3 min) were removed and stored at -80ºC until enzymatic analysis . Activity determinations were made using 40 µL of sample and 250 µL of NADH (0.24mM) and 40 µL of piruvate (10mM). The enzyme activity is expressed as unit (U) per mg of protein. A U is a µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.3$x10^{-3}$ M^{-1} cm^{-1}.

2.10 Acetylcholinesterase (AChE)

Using the data obtained from the cholinesterase characterization, we used the following procedure. The head was homogenized using a homogenizer or sonicator in 500 µl of potassium phosphate buffer (0.1M, pH 7.2), the supernatants obtained after centrifugation of the homogenates (4 ºC, 1 700 g, 3 min) were removed and stored at -80ºC until enzymatic analysis. The AChE activity determinations, was according to the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996)

In a 96 well microplate 250 µl of the reaction solution was added to 50 µl of the sample and absorbance was read at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280 ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzyme activity is expressed as unit (U) per mg of protein. A U is a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

2.11 Protein quantification for biomarkers

The protein concentration was determined according to the Bradford method (Bradford 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

2.12 Energy Reserves: Protein and Carbohydrate quantification

To determine total protein and carbohydrate content, isopods were homogenized using a homogenizer or a sonicator with a sonicator in 600 µl distilled water after which 200 µl of 15% trichloroacetic acid (TCA) was added and incubated at –20 ºC for 10 min. After centrifugation (1 000g, 10 min, 4ºC), the supernatant was separated has the carbohydrate fraction. The remaining pellet was resuspended in 2.5ml NaOH, incubated at 60 ºC for 30 min, after which it was neutralised with 1.5 ml HCl and used has the protein fraction.

Total protein content was then determined using Bradford's reagent (Bradford 1976), by measuring the absorbance at 590 nm using bovine serum albumin as a standard.

Total carbohydrate content was determined by adding 50 µl of 5% phenol and 200 µl H_2SO_4 to 50 µl of sample in a multiwell microplate, incubated for 30min at 20 ºC, and

the absorbance was measured at 492 nm using glucose as a standard. The protein and carbohydrate content is expressed as mg/ mg org and J/mg org (expressed as fresh weight).

2.13 Energy Reserves: Lipid quantification

Total lipid quantification was based in the method described by Bligh & Dyer (1959). Isopods were homogenized using a homogenizer or a sonicator in 200 µl double-distilled water after which 500 µl chloroform (spectrofotometric grade) were added. After vortexed more 500 µl methanol (spectrofotometric grade) and 250 µl double-distilled water were added to the previous content, centrifuged (1 000g, 5min, 4ºC) and the top phase removed; the remaing phase was used for lipid measurement. To 100 µl of lipid extract were added 500 µl H_2SO_4 and heated for 15 min (200ºC); after cooling down, 1.5 ml of double-distilled water was added and total lipid content was determined by measuring the absorbance at 370 nm using tripalmitin as a standard. The lipid content is expressed as mg/ mg org and J/mg org (expressed as fresh weight).

2.14 Chemical compounds

All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany) and were all of high quality and purity.

2.15 Statistics

Values for *in vitro* inhibition concentration (IC_{50}) were calculated using a nonlinear four parameter logistic curve for eserine hemisulfate and a nonlinear 2 parameters exponential decay curve for BW284C51 (SPSS 1999). An analysis of variance (ANOVA) was performed to compare differences between concentrations. Dunnett's comparison test was carried out to discriminate statistical different treatments (SPSS 1999). Comparisons between the two types of samples processing (homogenize vs sonicator) were made by using Students test (SPSS 1999), except in LDH were a Mann-Whitney Rank Sum Test was performed (SPSS 1999).

3 Results

3.1 Homogenization methodology

When we compare the two procedures for homogenization significant differences were found for the amount of extracted biomarkers protein (t_{18}= 5.959; p<0.001), GPx (t_{18}= -2.193; p=0.042), AChE (t_{18}= 7.872; p<0.001) and LDH (T=115; p<0.045). On the other hand, no differences were obtained on these procedures for CAT (t_{18}=-0.373; p=0.714), LPO (t_{18}=-1.325; p=0.202), GST (t_{18}=-0.581; p=0.568), carbohydrates (t_{18}=1.467; p=0.160), lipids (t_{17}=0.227; p=0.823) and proteins (T=111; p=0.094).

Therefore, all procedures used in the determination of all enzymatic activities depended on these results.

3.2 Cholinesterase characterization

To investigate the substrate preferences of ChE in the head tissues of *P. pruinosus*, three substrates were assayed: AcSCh, PrSCh, and BuSCh. ChE activity in the different head tissues as a function of increasing concentrations of substrates is presented in Fig. 7. Although the maximum activity of 201.94 (± 5.38 SE) U/mg protein was obtained with AcSCh at 10.24 mM, in the stable zone of the graph, we consider the value of 99.55 (± 3.24 SE) U/mg protein at 2.56 mM) obtained at the end of the exponential phase has the concentration to be used for future studies. Lower activities were observed when PrSCh value of 70.69 (± 3.16 SE) U/mg protein at 20.48 mM and BuSCh value of 2.80 (± 0.87 SE) U/mg protein at 20.48 mM were used as substrates.

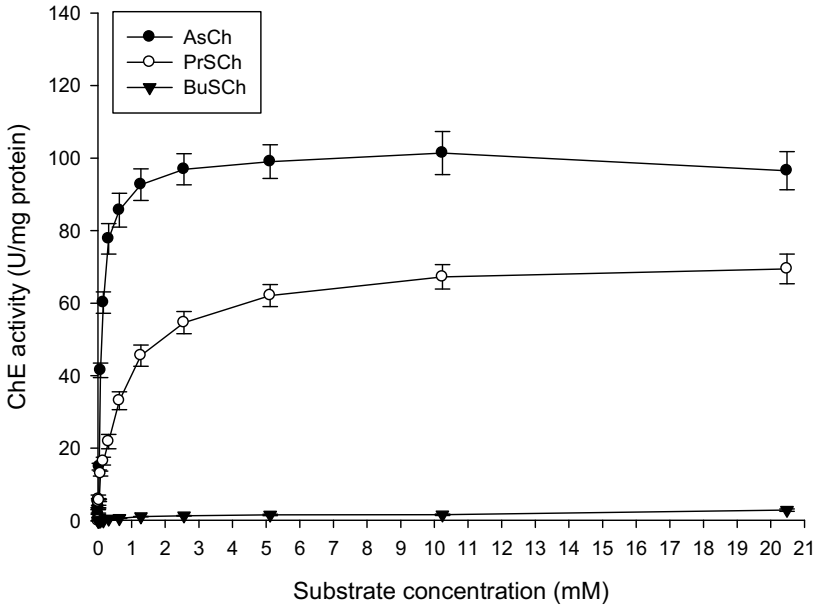

Fig. 7 ChE activity of *Porcellionides pruinosus* as a function of acetylthiocholine iodide (AcSCh), propionylthiocholine iodide (PrSCh) and *S*-butyrylthiocholine iodide (BuSCh) concentration. Values are means of 6 isopods' heads with 4 enzymatic determinations per isopod and the corresponding standard error bars.

The apparent K_m value for the AcSCh substrate calculated by the Lineweaver and Burk method was 356 μM (Fig. 8).

Eserine hemisulfate significantly inhibited ChE activity (p<0.001) (Fig. 9), and similar results were obtained with the selective inhibitor of AChE, BW284C51 (p<0.001), although data did not show a normal distribution (Fig. 10). Inhibition by eserine hemisulfate and BW284C51 was almost complete (>99%) at the highest concentrations tested. The effect of the selective inhibitor of BChE iso-OMPA did not affect *P. pruinosus* ChE activity (p>0.005) at concentrations up to 8 mM. IC_{50} values for eserine hemisulfate and BW284C51 are, respectively, 0.12 (± 3.22 SE) U/mg protein and 0.26 (± 0.06 SE) U/mg protein; IC_{50} values for iso-OMPA could not be determined since no significant inhibition was found up to the maximum concentration.

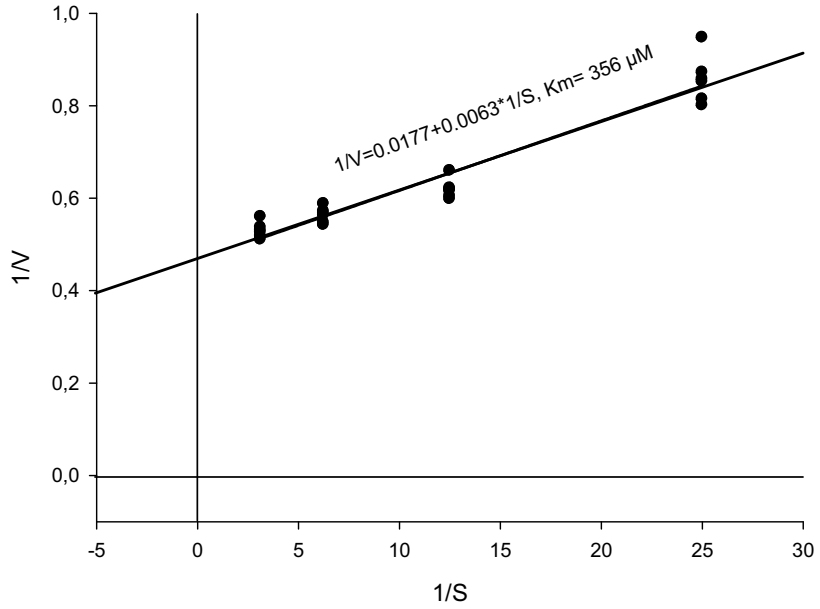

Fig. 8 Apparent K_m value for acetylthiocholine iodide (ASCh) substrate presented in a Lineweaver and Burk graph.

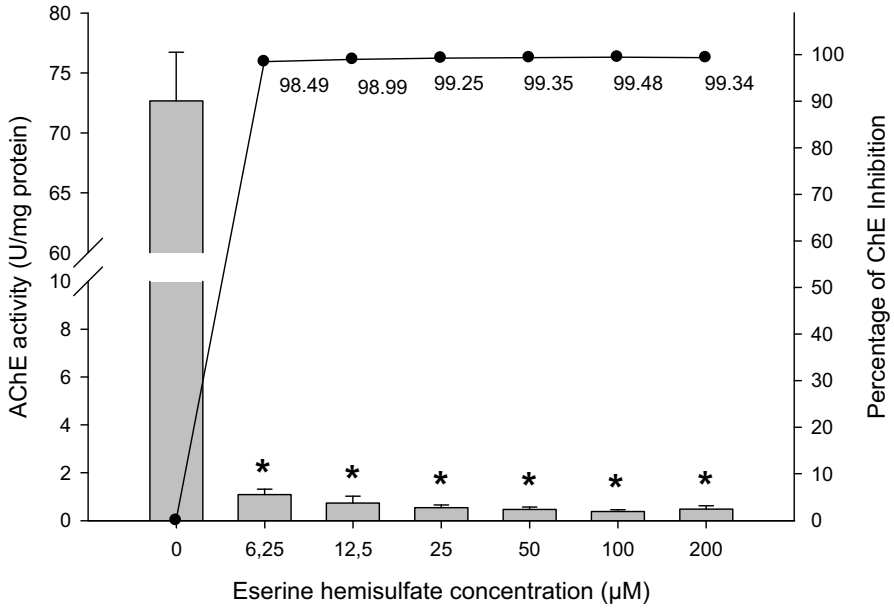

Fig. 9. ChE activity of *Porcellionides pruinosus* as a function of acetylthiocholine iodide (AcSCh), propionylthiocholine iodide (PrSCh) and *S*-butyrylthiocholine iodide (BuSCh) concentration. Values are means of 6 isopods' head with 4 enzymatic determinations per isopod and corresponding standard error bars. Bars correspond to AChE activity and the line to the percentage of ChE inhibition. *= Dunnett's test, $p<0.05$

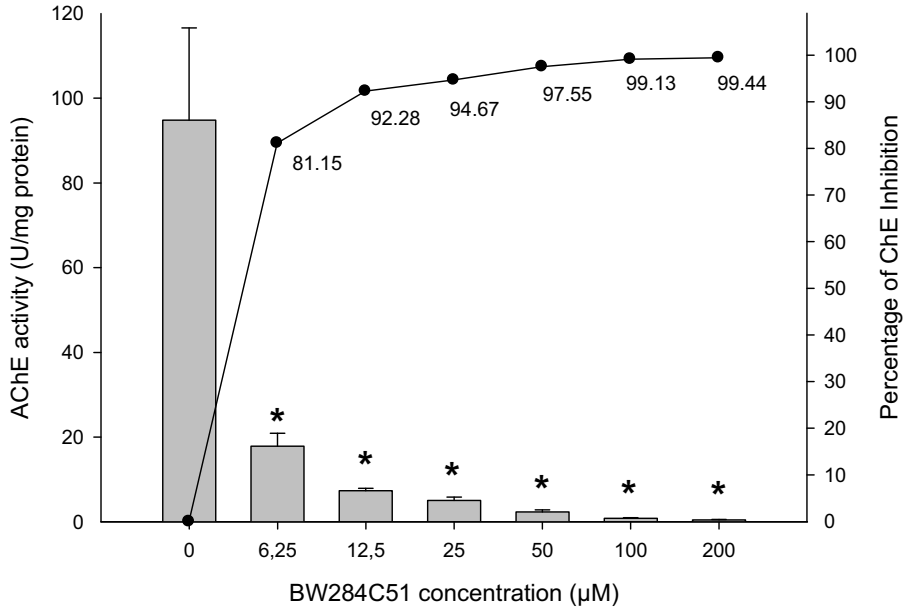

Fig. 10. Effect of BW284C51 on ChE activity of *Porcellionides pruinosus*. Values are mean of 6 isopods' head, with 4 enzymatic determinations per isopod and corresponding error bars. Bars correspond to AChE activity and the line to the percentage of ChE inhibition *= Dunnett's test, $p<0.05$

3.3 Normal range of biomarkers activity

Mean values for the basal levels of all molecular biomarkers measured are depicted in Table 1.

3.4 Energy reserves quantification

The mean carbohydrates and protein content analysed in *P. pruinosus* are reported in Table 2.

Table 1 Examples of biomarkers activities in several species used as test-organisms in ecotoxicological approaches. Values for this study on *Porcellionides pruinosus* are expressed as the mean value of 10 replicates with four enzymatic determinations per sample. Values for other species were reported in previous works, using here the activities obtained in the control's situations. SE- standard error; SD- standard deviation

	ACHE (U/mg protein)	LDH (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	LPO (U/mg ww)	GST (U/mg protein)
Porcellionides pruinosus (Sonicator)	113.56 ± 7.00 SE	3.03 ± 0.39 SE	6.11 ± 0.40 SE	2.73 ± 0.36 SE	34.57 ± 6.50 SE	137.76 ± 16.06 SE
Porcellionides pruinosus (Homogenizer)	52.05 ± 6.78 SE	5.15 ± 3.41 SE	6.38 ± 0.60 SE	4.12 ± 0.52 SE	47.53 ± 7.31 SE	119.47 ± 12.15 SE
Porcellio dilatatus (Ribeiro et al. 1999)	13.35 ± 2.52 SD	10.00 ± 4.14 SD	---	---	---	---
Mytilus galloprovincialis (Guilhermino et al. 1998)	68	---	---	---	---	---
Crangon crangon (Menezes et al. 2006)	72	0.0058	---	---	---	15.5
Pomatoschistus microps (Quintaneiro et al. 2008)	50.36 ± 1.39 SE	0.16 ± 0.01 SE	---	---	---	72.16 ± 4.69 SE
Daphnia magna Clone S-1 (Barata et al. 2001)	62.3	---	---	---	---	---
Daphnia magna Clone F (Barata et al. 2001)	14.9	---	---	---	---	---
Daphnia magna Clone A (Barata et al. 2001)	27.2	---	---	---	---	---
Daphnia magna Clone 13 (Barata et al. 2001)	28.4	---	---	---	---	---
Daphnia magna Clone 9 (Barata et al. 2001)	36.7	---	---	---	---	---
Porcellio scaber (Stanek et al. 2006)	320	---	---	---	---	---
Enchytraeus albidus (referencia)	---	---	20	4.5	80	14
Porcellio scaber (Drobne et al. 2008)	---	---	---	---	---	1400
Porcellio scaber (Drobne et al. 2009)	---	---	13	---	---	250
Daphnia magna (De Coen et al. 2006)	---	0.5	---	---	---	---
Danio rerio (larvae) (Oliveira et al. in press)	90	0.22	---	---	---	20
Danio rerio (adult) (Oliveira et al. in press)	125	0.4	---	---	---	45

Table 2 Examples of energy reserves content in several species used as test-organisms in ecotoxicological approaches. Values for this study on *Porcellionides pruinosus* are expressed as the mean value of 10 replicates and corresponding error. Values for other species were reported in previous works, using here the activities obtained in the control's situations. SE- standard error

	Carbohydrates		Proteins		Lipids	
	(mJ/mg org.)	(µg/ mg org.)	(mJ/mg org.)	(µg/ mg org.)	(mJ/mg org.)	(µg/ mg org.)
P. pruinosus (Sonicator)	218.17 ± 30.95 SE	12.47 ± 1.77 SE	872.14 ± 30.23 SE	36.34 ± 1.26 SE	866.48 ± 63.91 SE	22.72 ± 1.46 SE
P. pruinosus (Homogenizator)	162.73 ± 35.28 SE	9.30 ± 2.02 SE	914.13 ± 38.36 SE	39.17 ± 1.60 SE	848.17 ± 46.56 SE	21.47 ± 1.79 SE
Porcellio dilatatus (Calhôa Unpublished data)	90.74 ± 8.45 SE	5.18 ± 2.02 SE	559.54 ± 9.80 SE	23.31 ± 2.00 SE	429.48 ± 10.70 SE	10.87 ± 1.70 SE
Porcellio scaber (Stanek et al. 2006)	---	0.9	---	55	---	0.9

4 Discussion

Sample's preparation appears to be a very important step in the measurement of biomakers activity. When applying two different methodologies for the homogenization procedure (homogenizer and sonicator), there were significant differences in some of the analysis. Although in some case lower enzymatic activities were determined, the standard error associated with samples where sonication was used were much lower then the ones obtained by using the homogenizer. Another fact that supports the decision of choosing the sonicator over the homogenizer is the amount of protein obtained when using a sonicator or a homogenizer which were respectively 3.34 ± 0.09 SE mg of protein and 2.75 ± 0.05 SE mg of protein (t_{18}= 5.959; p<0.001).

One of the objectives of this study, the characterization of the ChE activity in *P. pruinosus*, included a first step to distinguish ChE from nonspecific esterases. This procedure is important because tissues may contain several nonspecific esterases, which contribute to the measured activity and may show different sensitivities towards anticholinesterase agents (Garcia et al. 2000). Nonspecific esterases contribution was estimated using the compound eserine hemisulfate, which is considered a specific inhibitor of ChE at low concentrations, in the 10^{-6} - 10^{-5} M range (Eto 1974). In the present study the enzymatic activity measured was almost full inhibited by eserine hemisulfate at 6.25 μM (98.49%). This result was found indicating that the enzymes are predominantly from ChE and not from other esterases.

The highest ChE activity of *P. pruinosus* was obtained with AcSCh, showing a distinct preference over the other substrates. Furthermore, there was an almost complete inhibition when BW284C51 was used, while no significant inhibition was observed with iso-OMPA. Thus it seems that the main form present in this species is AChE. Since there are no more works associated with the characterization of ChE on isopods no comparisons could be made.

The K_m value obtained was significantly higher than the ones published for other invertebrates such as the fall armyworm *Spodoptera frugiperda*, 33.5 μM (Yu 2006), or the mollusc bivalve *Mytilus galloprovincialis*, 34 μM (Mora et al. 1999), but similar to

the earthworm *Eisenia Andrei*, 160 µM (Gambi et al. 2007), and the mollusc bivalve *Pecten jacobaeus*, 274.8 µM in gills and 233.9 µM in the adductor muscle (Stefano et al. 2008).

The comparison of the determined basal levels on biomarkers activities and energy reserves with previous works shows some differences when comparing to other isopod species or other organisms (Table 1). When we compare AChE, GST, GPx activities within species, similar values are found, but other biomarkers like the LPO, CAT and LDH, some differences were found even within isopod species. As for the energetic reserves, all contents seems to be the double values when compared with the organism *Porcellio dilatatus*. When values from this study are compared to those obtained with for the organism *Porcellio scaber*, our organism show1.5x lower protein content, 12x and 24x higher content for carbohydrates and lipids respectively.

This study will be used as a foundation for future studies on the evaluation of biomarkers in the species *Porcellionides pruinosus* exposed to xenobiotics in the laboratory, but also as a possible biomonitorization tool for *in situ* testing..

Acknowledgment
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CHAPTER III

Effects of zinc and diazinon on biomarkers of Porcellionides pruinosus

Effects of zinc and diazinon on biomarkers of *Porcellionides pruinosus*

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Abstract:

In the last decades studies on biomarkers responses have been used to evaluate the effects of xenobiotics in the environment. But few data has been focused on this assessment tool using detritivorous key-organisms like isopods.

In this work the isopod *Porcellionides pruinosus* was exposed to contaminated food with zinc sulphate and the pesticide diazinon. The concentrations used were previously identified in other studies as NOEC and LOEC values and correspond to 5.5 and 9.5 µg zinc/ g dry leaf and 17.5 and 175 µg diazinon/ g dry leaf respectively. Biomarkers were tested and chemicals mode of action were evaluated considering their response patterns, along with the identification of which biomarker could give early warnings.

Biomarkers tested were acetylcholinesterase (AChE), lactate dehydrogenase (LDH), catalase (CAT), glutathione *S*-transferase (GST), glutathione peroxidase (GPx) and lipid peroxidation (LPO). The present study also presents for each contaminant which are the biomarkers that can provide better information of its action from the most to the less important, when assessing metal contamination (CAT, GST, LPO, GPx, AChE and LDH) and pesticide contamination (AChE, GPx, GST, LDH and CAT)

Keywords: biomarkers, isopods, zinc, diazinon

1 Introduction

Nowadays with new legislation for chemical testing like REACH or even with the need to remediate, prevent or early detect deleterious effects to the environment, new and accurate tools are needed.
Soil is seriously affected by xenobiotics and with the increase of pollution, key soil-dwelling organisms like terrestrial isopods are potentially at risk. Two classes of major xenobiotics affecting soil are metals and pesticides and their impact in the environment should be cautiously considered.

Terrestrial isopods are successful invaders of the terrestrial habitats, and are essential to the ecosystem functioning. They are macrodecomposers, feeding mainly on decaying plant material, and they play an important role in the detritus food chain, through litter fragmentation and stimulating and/or ingesting fungi and bacteria that are important in the cycling of nutrients (Loureiro et al. 2006). Due to their role and life traits they are strongly affect not only by the soil contaminants, but also by the contaminants present in litter-layer (Vijver et al. 2006).

Biomarkers, which can be described as any biological response to an environmental chemical below-individual level (vanGestel & vanBrummelen 1996) come associated to a sensitivity and quick response that may give early alarms signals in organisms, well before ecological disturbances can be observed (Morgan et al. 1999). For this reason using biomarkers as an ecotoxicological tool one will possibly and easily link information on the presence of a pollutant (or pollutant class) and also on the effects to organisms. This approach will also enable to assess early changes on organisms' fitness, at organizational levels below the individual that can not become apparent in other types of tests (Kammenga et al. 2000, vanGestel & vanBrummelen 1996).

But the use of biomarkers on isopods and the creation of ecotoxicological tests based on them need a previous determination of the patterns of response when exposed to different types of xenobiotics.

Zinc is one of the essential trace elements for the nutrition, structural, catalytic and regulatory functions in organisms, but can become toxic when its concentration in an

organism seriously exceeds physiological limits (Drobne & Hopkin 1995, Jones & Hopkin 1998).

Diazinon is a nonsystemic organosphosphate insecticide and acaricide developed in the early 1950s and it is used to kill insects by inhibiting acetylcholinesterase, an enzyme necessary for proper nervous system function.

The objectives of this study were: i) to determine the response pattern of the biomarkers acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione *S*-transferase (GST), catalase (CAT), lipid peroxidation (LPO), glutathione peroxidase (GPx) when the terrestrial isopod *Porcellionides pruinosus* was exposed to food contaminated with zinc sulphate and diazinon; ii) to compare the results with previous published studies and iii) determine which biomarkers can give early warning responses for metal and/or pesticide exposure.

2 Materials and methods

2.1 Test Organism and Culture Procedure

The organisms used in these experiments belong to the specie *Porcellionides pruinosus* (Brandt, 1833), and were previously collected from horse manure pills and maintained for several generations in laboratory cultures. In this cultures isopods are fed *ad libidum* with alder leaves (*Alnus glutinosa*) and maintained at 25 ± 2°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water spayed and extra food is provided. Only adult animals (15-25 mg wet weight) were used in the experiments; there was no distinction between sexes, although pregnant females were excluded.

2.2 Experimental procedure

In these experiments two plastic containers (Ø 80 mm; 120 mm high), one placed within the other were used (Fig. 11). The upper box had a net bottom to allow faeces to pass to the box below, which had a plaster bottom to provide a constant moist environment. The upper box was sealed with parafilm and holes were made to ensure ventilation. Test animals were collect from culture box, weighted (15-25mg) and placed individually in each test-box (upper part) with the contaminated leaf material. Animals with

abnormalities, moulting or pregnant females were discarded. The boxes were placed in a climate chamber at 25°±2°C, with a 16h:8h (light-dark) photoperiod.

Experiments were divided in two sets that lasted for 96 hours and 7-days. Every day, test-containers were checked for dead animals and if necessary water was sprayed on the plaster bottom to ensure a constant moist environment.

Biomarker assays were performed, using a pool of two organisms for glutathione *S*-transferase (GST), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO). One organism was used and divided into head and body to test acetylcholinesterase (AChE) and lactate dehydrogenase (LDH), respectively.

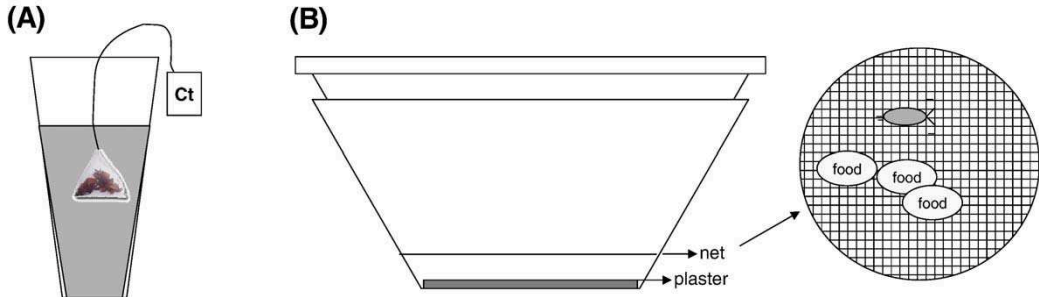

Fig. 11 Scheme for leaf contamination (A) and experimental test boxes (B) (Loureiro et al. 2006)

2.3 Leaf contamination

For the zinc sulphate exposure test, alder leaves were cut as disks (Ø 10 mm) and weighed (± 20 mg), enclosed in a net bag and submerged in the contaminated solution for 4 days. The concentrations of contaminant used were 20 and 100 mg/L to achieve around 5.5 and 9.5 µg Zn/g of leaf (Loureiro et al. 2006), and for the control, leaf disks were submerged in distilled water. For each concentration ten replicates were prepared. In the beginning of the experiments leaf disks were removed from the net bags, air dried and placed in the upper test boxes.

For the diazinon exposure test, a stock solution was made in ethanol and the concentration range in double-distilled water. The concentrations of contaminant used were 17.5 and 175 µg diazinon per gram dry food, and for the control, leafs disks were

moistened with distilled water. Alder leaf disks were contaminated on the day of use and topically.

The final leaf concentration of 5.5 and 9.5 µg zinc / gram dry food has been based on the findings of (Loureiro et al. 2006). These values are the NOEC and LOEC values, respectively for the consumption ratio when exposed to the same conditions. The final leaf concentration of the pesticide diazinon (17.5 and 175 µg diazinon / gram dry food) has also been considered by (Vink et al. 1995) as NOEC and LOEC respectively on the energy budget of *P. pruinosus* when exposed to contaminated food.

2.4 Post-mitochondrial supernatant (PMS)

Each replicate composed of two organisms were sonicated in 1 ml K-Phosphate 0.1 M buffer, pH 7.4. From the homogenate 300 µL were separated into a microtube and 5 µL butylated hydroxytoluene (BHT) 4% in methanol were added for endogenous lipid peroxidation (LPO) determination. The remaining tissue homogenate (700 µL) was centrifuged at 10 000g for 20 min. (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was divided into four microtubes for posterior analysis of biomarkers and protein quantification. All microtubes were stored at -80ºC until analysis, for a period no longer than 1 week.

2.5 Lipid peroxidation (LPO)

The lipid peroxidation (LPO) assay was based on the method described by Bird & Draper (1984), Ohkawa et al. (1979) by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 300 µL homogenated tissue, 1 mL TCA 12% (w/v), 1 mL TBA 0.73% (w/v) and 800 µL Tris-HCl 60mM with DTPA 0.1 mM. The reaction mixture was then incubated at 100ºC in a water bath for 1h. After this, tubes were centrifuged for 5 min. at 11 500 rpm (25ºC). Samples were kept away from light, at 25ºC and immediately read at 535 nm. The enzyme activity is expressed as unit (U) per mg of wet weight. A U is a nmol TBARS hydrolyzed per minute, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

2.6 Glutathione S-Transferase (GST)

Glutathione *S*-Transferase (GST) activity was determined based on the method described by Habig et al. (1974) and adapted to microplate (Diamantino et al. 2001). We mixed 100 µL of PMS in 200 µL of a reaction solution. The reaction solution was a mixture of 4,950 ml K-Phosphate 0.1 M (pH 6.5) with 900 µL GSH 10mM, and 150 µL 1-chloro-2,4- dinitrobenzene (CDNB) 10mM. It was measured at 340 nm. The enzyme activity is expressed as unit (U) per mg of protein. A U is a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6 $x10^{-3}$ M^{-1} cm^{-1}.

2.7 Glutathione Peroxidase (GPx)

Glutathione Peroxidase (GPx) activity was determined based on the method described by Mohandas et al. (1984). We mixed 50 µl PMS with 840 µl K-Phosphate 0.05 M (pH 7.0) with EDTA 1 mM, Sodium azide 1 mM and GR (7.5 mL from stock with 1 U/mL), and 50 µL GSH 4 mM, by measuring the decrease in NADPH (50 µl, 0.8 mM) at 340 nm and using H_2O_2 (10 µl, 0.5 mM) as substrate (Mohandas et al. 1984). The enzyme activity is expressed as unit (U) per mg of protein. A U is a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.22$x10^{-3}$ M^{-1} cm^{-1}.

2.8 Catalase (CAT)

Catalase (CAT) activity was determined based on the method described by (Clairborne (1985). We mixed 50 µl of PMS with 500 µL H_2O_2 0.030 M, and 950 µL K-Phosphate 0.05 M (pH 7.0) and measured the decomposition of the substrate (H_2O_2) at 240 nm. The enzyme activity is expressed as unit (U) per mg of protein. A U is a µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

2.9 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) activity was determined at 340nm by the method of Vassault (1983) adapted to microplate by Diamantino et al. (2001). Whole body was sonicated in 500 µl of TRIS/NACl buffer (0.1M, pH 7.2), the supernatants obtained after centrifugation of the homogenates (4 ºC, 1 700 g, 3 min) were removed and stored at -80ºC until enzymatic analysis . Activity determinations were made using 40 µL of sample and 250 µL of NADH (0.24mM) and 40 µL of piruvate (10mM). The enzyme

activity is expressed as unit (U) per mg of protein. A U is a μmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $6.3x10^{-3}$ M^{-1} cm^{-1}.

2.10 Acetylcholinesterase (AChE)

Using the data obtained from the cholinesterase characterization, we used the following procedure. The head was sonicated in 500 μl of potassium phosphate buffer (0.1M, pH 7.2), the supernatants obtained after centrifugation of the homogenates (4 ºC, 1 700 g, 3 min) were removed and stored at -80ºC until enzymatic analysis. The AChE activity determinations, was according to the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996)

In a 96 well microplate 250 μl of the reaction solution was added to 50 μl of the sample and absorbance was read at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280 ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzyme activity is expressed as unit (U) per mg of protein. A U is a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

2.11 Protein quantification for biomarkers

The protein concentration was determined according to the Bradford method (Bradford 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

2.12 Chemical compounds

All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany) and were all of high quality and purity.

2.13 Statistics

One-way analysis of variance (ANOVA) using the SigmaStat statistical package (SPSS 1999) was used to test for statistical differences between concentration treatments. Whenever significant differences were found a Dunnett's comparison test was performed. Whenever data were not normally distributed and data transformation did

not correct for normality, a Kruskal Wallis ANOVA on Ranks was performed, followed by the Dunnett's or Dunn's method when significant differences were found.

3 Results

3.1 Zinc sulphate exposure: biomarkers activity

The results obtained for the acetylcholinesterase (AChE) activity *P. pruinosus* exposed for 96h to zinc sulphate (Fig. 12) showed a non-significant activity increase for 5.5 µg/ mg dry leaf, and a statistical significant decrease for 9.5 µg/ mg dry leaf (Dunnett's test $p<0.005$). After a 7-day exposure period an increase was observed for both concentrations, but only significant for the 5.5 µg/ mg dry leaf (Dunnett's test $p<0.005$).

For the lactate dehydrogenase (LDH) no significant differences were found. Even though an increase for NOEC was observed at 96h, decreasing after the 7-day exposure period.

The glutathione *S*-transferase (GST) activity showed a non-significant increase with the increase of concentrations for the 96h exposure period, and a significant activity inhibition with increasing concentrations after the 7day period of exposure (Dunnett's test $p<0.005$).

The catalase (CAT) activity showed an increasing inhibition with increasing concentrations when compared with the control in both exposure periods, being this inhibition significant only for the 9.5 µg/ mg dry leaf concentration (Dunnett's test $p<0.005$).

For the lipid peroxidation (LPO) biomarker, it was quantified an increase for the 96h and 7-day exposure period being it significant for the 9.5 µg/ mg dry leaf at a 7-day exposure period (Dunnett's test $p<0.005$).

The glutathione peroxidase (GPx) activity shows a non significant inhibition when compared with the control for all the concentrations for both 96h and 7-day exposure period.

A Two-Way ANOVA showed a significant interaction between concentrations and time for exposure for AChE (p=0.003), GST (p=0.013), LPO (p=0.012), and no interaction for LDH (p=0.542), CAT (p=0.078) or GPx (p=0.414).

When we compare the controls for time exposure significant differences were found only for AChE (t_{17}=-2.159; p=0.045).

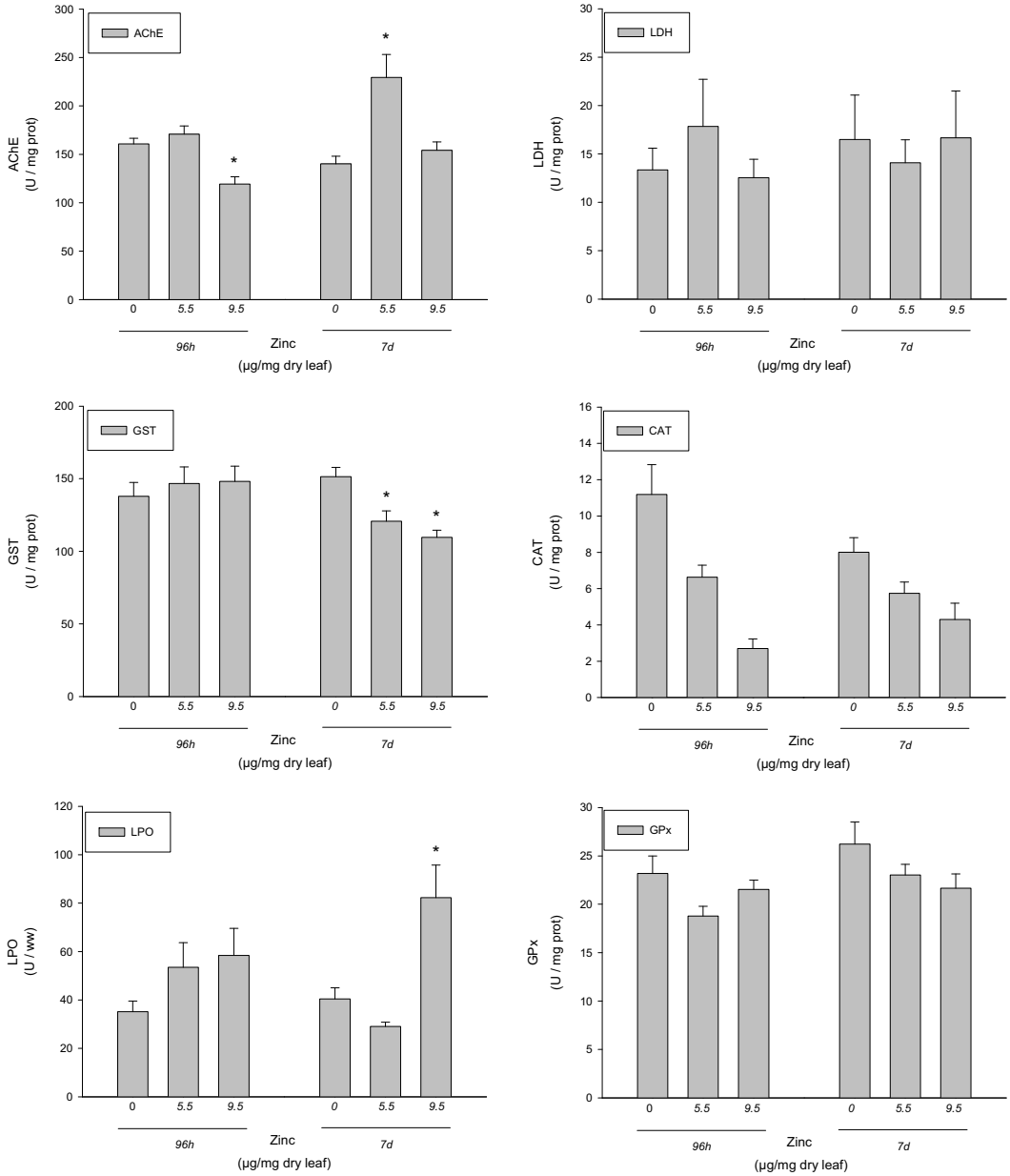

Fig. 12. Results of acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione *S*-transferase (GST), catalase (CAT) and glutathione peroxidase (GPx) activity for *Porcellionides pruinosus* when exposed to zinc sulphate. Bars are mean values and corresponding standard error bars. ***=** Dunnett's test, p<0.05

3.2 Diazinon exposure: biomarkers activity

The results obtained for *P. pruinosus* when expose to diazinon are shown in Fig. 13. For acetylcholinesterase (AChE) it depicts an increase in the activity when compared with the control for both time of exposures, being this increase significant for 175 µg/g dry leaf at 96h exposure period (Dunnett's test $p<0.005$) and for 17.5µg/g dry leaf at 7-day exposure period (Dunnett's test $p<0.005$).

For the lactate dehydrogenase (LDH) no significant differences were found, although a slight inhibition was observed at 96h exposure period, contrary to the slight increase observed at a 7-day exposure period.

The glutathione *S*-transferase (GST) activity showed no significant differences for both concentrations at both exposure periods.

The catalase (CAT) activity for both exposure periods showed a non-significant inhibition when compared with the control, although a sight inhibition was observed for both exposure periods.

The glutathione peroxidase (GPx) activity increased with concentration and time of exposure period, but was only significant for 175µg/g dry leaf at a 7-day exposure period (Dunnett's test $p<0.005$).

A Two-Way ANOVA showed a significant interaction between concentrations and time for exposure for AChE ($p<0.001$) and GPx ($p<0.001$), and no interaction for GST ($p=0.132$), LDH ($p=0.054$), CAT ($p=0.433$).

When we compare the control for time exposure significant differences were found only for AChE ($t_{16}= -2.739$; $p<0.015$) and LDH ($t_{13}= 2.247$; $p<0.043$).

Due to an unknown error the LPO for the diazinon exposure could not be determined.

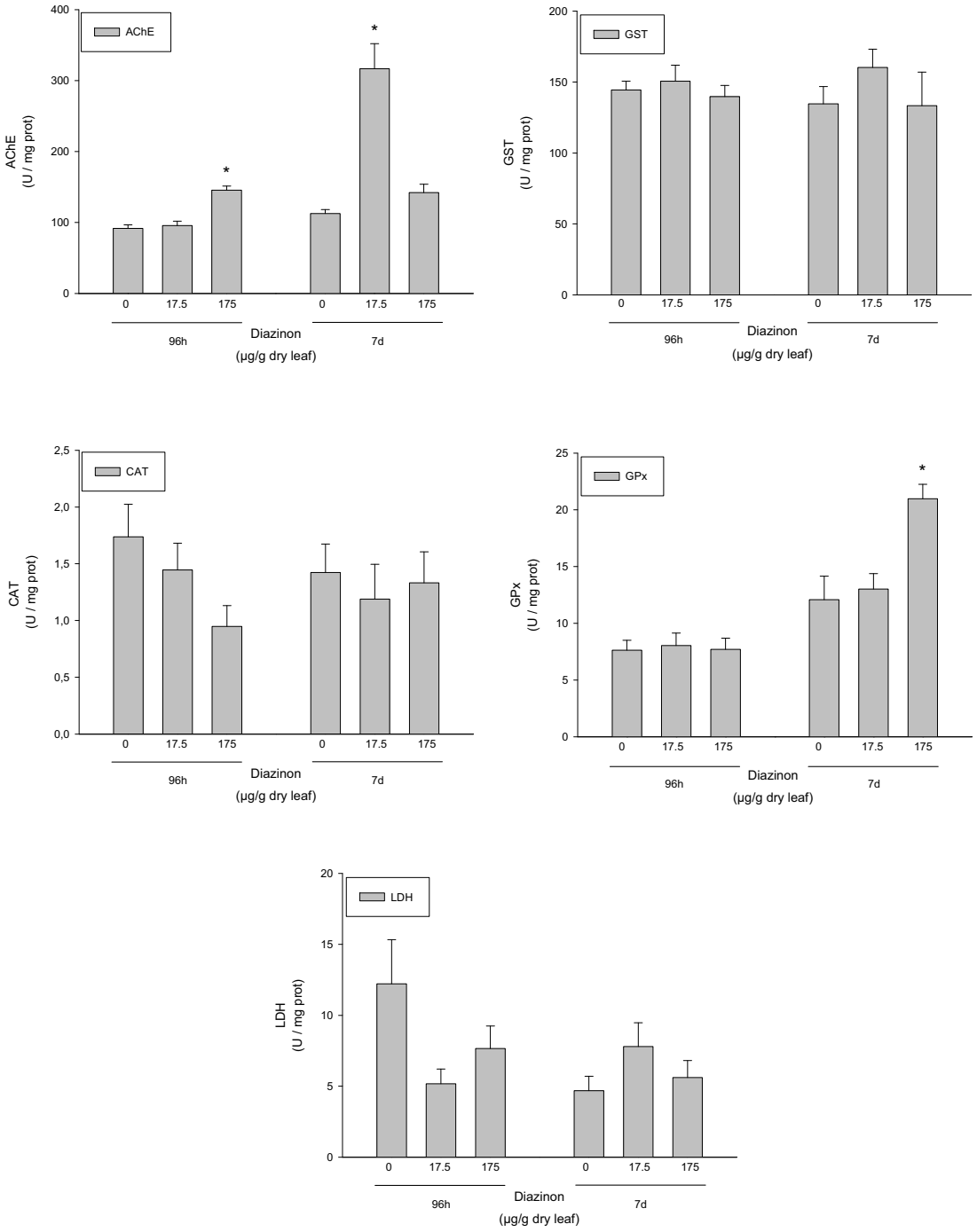

Fig. 13 Results of acetylcholinesterase (AChE), lactate dehydrogenase (LDH), glutathione S-transferase (GST), catalase (CAT) and glutathione peroxidase (GPx) activity for *Porcellionides pruinosus* when exposed to diazinon. Bars are mean values and corresponding standard error bars. *= Dunnett's test, $p<0.05$

4 Discussion

The present work showed the response of biomarkers when exposed to two different contaminants, the metal zinc and the pesticide diazinon and the biomarkers that could give early warnings about the organism status not only with small exposure periods, but also using concentrations that have previously showed no effect on organisms, when ecotoxicological testing was carried out. In literature it is well described the effects of each of the contaminants on isopods. Zinc is known to affect the activity of metal-binding enzymes, and diazinon is known to play a strong influence in the biomarker AChE because it was designed to affect its activity.

The comparison of the control activities for both time exposure periods in almost all cases showed non-significant differences, with the exception of AChE (t_{17}= 2.159; p=0.045) when exposed to zinc and of AChE (t_{16}= -2.739; p=0.015), LDH (t_{13}= 2.247; p=0.043) when exposed to diazinon. This significant differences can be a result of other extra stress factors not related to the contaminant exposure. For these reason the results obtained for these enzymes should always be carefully analysed.

When analysing the activity of biomarkers when organisms are exposed to zinc sulphate the lactate dehydrogenase (LDH) seems to be the less sensitive and accurate biomarker to use as no significant differences were determined.

The glutathione peroxidase (GPx) like the LDH biomarker, does not present any significant difference for both concentrations and exposure periods, a non expected decreasing pattern can be observed for a 7-day exposure period. This result can not be interpreted by itself since glutathione (GSH) is used by this enzyme to transform H_2O_2 into oxidized glutathione (GSSG) and no quantification of GSSG or glutathione reductase (GR) have been done in this work.

As for the glutathione *S*-transferase (GST), an increase on its activity should be expected since it is one of the most important phase II group enzymes and is responsible for the increase of the availability of lipophilic toxicants to phase I enzymes (Schelin et al. 1983). The 96h exposure period show a non-significant increase in the activity, but after the 7-day exposure period it shows a significant decrease in the enzymatic activity

for both concentrations, contrasting with the described literature. The explanation for the decrease in the GST activity can be the same as the one for the GPx since GSH is also needed for its oxidative stress action.

Catalase is a metal-biding enzyme, and its activity decreases along with zinc increasing concentration observing a significant inhibition by the 9.5 µg/ mg dry leaf concentration for both exposure periods.

The lipid peroxidation (LPO) shows an increasing pattern for both exposure periods, being significant only after a 7-day period.

For last the acetylcholinesterase shows an increasing pattern for the 5.5 µg/ mg dry leaf, being significant at a 7-day exposure period and an inhibition for the 9.5 µg/ mg dry leaf at 96h that the organisms seem to recover at a 7-say period, even increasing their activity when comparing to the control.

The result obtained for CAT and GPx contradicts the ones found for *Enchytraeus albidus* exposed to copper (Howcroft et al. 2009), where an increase was observed for a 96h and 3 weeks exposure period.

When comparing our results with the ones obtained for Loureiro et al. (2006) where the same specie (*Porcellionides pruinosus*) was exposed for a 14-day period to zinc sulphate, a correlation between the food consumption and biomarkers activity can be made using the data from both works. So the decrease in food consumption observed for the 9.5 µg diazinon/g dry leaf concentration can maybe be explained by a significant decrease in the activity of the biomarkers GST and CAT, along with an increase of the LPO observed for a 7-day exposure period. The same pattern of these biomarkers activity is observed for the 5.5 µg diazinon/g dry leaf concentration, where non-significant differences are found for both works, supports the previous result.

When the isopod *P. pruinosus* was exposed to the pesticide diazinon, biomarkers activity showed a different pattern from the ones described above. Starting by the AChE where a strong effect should be noticed, in the 96h only the 175 µg/g dry leaf concentration show a significant activity increase, although an increase for the NOEC is

already observed. After a 7-day exposure period the 17.5 µg/g dry leaf concentration shows twice the activity observed for the control, which can indicate that long time exposure periods can be harmful to this organism. The 175 µg/g dry leaf concentration at a 7-day exposure period seems to be the same for the 96h exposure period, that analysed along with the 17.5 µg/g dry leaf concentration activity can be explained has an inhibition after the enzyme reach the maximum activity in a period between the 96h and 7-day sampling period. The response observed for AChE was not the one expected since a previous work from Stanek et al. (2006) showed that the isopod *Porcellio scaber* when exposed to diazinon with a range from 0-100 µg/g dry leaf concentration always presented a decrease in the AChE activity.

The LDH response for diazinon exposure seems to be the opposite found for the zinc exposure, although no significant differences in activity were found, the NOEC seems to decline at a 96h period and increase at a 7-day period.

Both GST and GPx activities of isopods exposed to diazinon increased for both concentrations and along with time, having a significant activity increase of GPx for 175 µg/g dry leaf concentration at a 7-day exposure period, contrary to the described in literature for the rainbowtrout (*Oncorhynchus mykiss*) when exposed to diazinon (Isik & Celik 2008)

The CAT enzyme shows no variation for both concentrations and exposure periods, which indicates that this enzyme may not be involved in the detoxification of the pesticide diazinon.

When comparing our results with the ones obtained for Vink et al. (1995) where the same specie (*Porcellionides pruinosus*) was exposed for a 6 week period to diazinon, only a correlation between the energy reserves content and the biomarkers GPx activity could be established. A decrease in carbohydrates and lipid content could maybe be explained by a significant increase in the activity of the biomarkers GPx observed for a 7-day exposure period.

It is important to take into consideration that almost all biomarkers showed an interaction between concentration and time of exposure, which means that patterns

observed both in after 96h and/or 7-day of exposure that are not significant can become significant in a long-term exposure.

As described above some biomarkers can give early warnings of an organism abnormal status even when exposure concentrations are considered as no effect concentrations. Based on the results a gradient of more to less important biomarker can be described for each one of the contaminants, although the use of one biomarker should not be carried out individually excluding other since none alone can give the real status of the organism. So for the exposure to zinc sulphate biomarkers that respond better insight to chemical exposure were the GST, CAT activity and the LPO. For the pesticide diazinon exposure biomarkers did not produce an accurate or expected result. GPx activity gave a clear response after 7 days of exposure and AChE activity was enhanced by diazinon exposure. The other biomarkers did not respond to the concentrations used.

To complement this study other biomarkers activity should be quantified such as the glutathione reductase (GR), superoxide dismutase (SOD) and the non enzymatic biomarkers glutathione reduced (GSH) and glutathione oxidized (GSSG), can maybe explain some adverse patterns observed for enzymes like GST and GPx in the zinc exposure, or even explain why no variation in some biomarkers activity are observed.

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Chapter IV

Effects of Zinc and Diazinon on the energy budget of the isopod Porcellionides pruinosus

Effects of Zinc and Diazinon on the energy budget of the isopod *Porcellionides pruinosus*

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Abstract:

The quantification of energy reserves: lipids, proteins and carbohydrates have been used for several years to assess contaminant impacts on organisms. Although results could be used for determining several ecotoxicological parameters more accurate results can be obtained by also determine the energy consumption and the integration of both the available and consumed energy.

In this work we quantify energy reserves, the electron transport system activity (ETS) and cellular energy allocation (CEA) in the isopod *Porcellionides pruinosus* exposed to food contaminated with zinc sulphate and the pesticide diazinon to identify the mode of action of the contaminants within each energy reserve. The concentrations use were identified in previous studies as NOEC and LOEC values and correspond to 5.5, 9.5 µg zinc/ g dry leaf and 17.5, 175 µg diazinon/ g dry leaf respectively.

The current work show no changes in lipid and protein content for isopods exposed for both the contaminants. And significant decrease in carbohydrates content for both concentrations at both exposure time when isopods were exposed to zinc sulphate and for both concentrations at a 14d-ay exposure period when isopods were exposed to diazinon. For zinc sulphate exposure is also observed a decrease in the highest concentration for a 14-day exposure period and also a decrease for CEA in both concentrations in the 7-day exposure period and dor the highest concentration in the 14day exposure period. No changes in ETS or CEA were observed for diazinon exposure.

The work showed that the quantification of the ETS activity and the CEA along with the energy reserves (lipids proteins and carbohydrates) is important when evaluating the effects of organisms, giving information on their fitness.

Keywords: energy reserves, CEA, isopods, zinc, diazinon

1 Introduction

Woodlice are important key soil-dwelling organisms responsible for macrodecoposing decaying plant litter into fragments and stimulating and/or ingesting fungi and bacteria that are important in the cycling of nutrients (Loureiro et al. 2006). Due to their role within the environment they are strongly affected not only by the soil contaminants, but also by the contaminants present in litter-layer where they fed (Vijver et al. 2006). These contaminants can induce changes on the concentration of stored energy reserves which are important for the maintenance, growth and reproduction requirements of any organism.

Energy reserves are normally stored has glycogen and lipids and are used whenever necessary, but under severe stress conditions proteins can also be used as energy reserves.

The quantification of energy reserves as an endpoint to assess deleterious effects of contaminants has been used by several authors (i.e. Staempfli et al. (2007), Van Brummelen & Stuijfzand (1993)), but only few authors have conjugated this quantification with electron transport system activity (ETS) and with the cellular energy allocation (CEA) (De Coen & Janssen 1997, De Coen et al. 1995, De Coen et al. 2001, Verslycke et al. 2004). The ETS can give information about the energy consumption (E_c) of the organism under stress conditions and when combined with the whole-body caloric content (transforming the lipid, protein and carbohydrate into energy) can give us a quantification for the allocation of cellular energy (CEA). CEA is based on the energy reserves available (E_a) and energy consumption (E_c) and is presented has a general stress index.

The main aim of this study was to evaluate the effects of zinc and diazinon on the energy reserves of *Porcellionides pruinosus*. For that, in this study we quantified the lipid, protein and carbohydrate contents along with ETS and CEA for exposed organisms and compared the results with previous published studies. Finally, we aimed to describe how each contaminant affects the organisms' energy budget.

2 Materials and methods

2.1 Test Organism and Culture Procedure

The organisms used in these experiments belong to the specie *Porcellionides pruinosus* (Brandt, 1833), and were previously collected from horse manure pills and maintained for several generations in laboratory cultures. In this cultures isopods are fed *ad libidum* with alder leaves (*Alnus glutinosa*) and maintained at 25 ± 2°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water spayed and extra food is provided. Only adult animals (15-25 mg wet weight) were used in the experiments; there was no distinction between sexes, although pregnant females were excluded.

2.2 Experimental procedure

In these experiments two plastic containers (Ø 80 mm; 120 mm high), one placed within the other were used (Fig. 14). The upper box had a net bottom to allow faeces to pass to the box below, which had a plaster bottom to provide a constant moist environment. The upper box was sealed with parafilm and holes were made to ensure ventilation. Test animals were collect from culture box, weighted (15-25mg) and placed individually in each test-box (upper part) with the contaminated leaf material. Animals with abnormalities, moulting or pregnant females were discarded. The boxes were placed in a climate chamber at 25°±2°C, with a 16h:8h (light-dark) photoperiod.

Experiments were divided in two sets that lasted for 96 hours and 7-days. Every day, test-containers were checked for dead animals and if necessary water was sprayed on the plaster bottom to ensure a constant moist environment.

To quantify the energy reserves (lipids, carbohydrates and proteins) one organism was used for carbohydrates and proteins and another one for lipids.

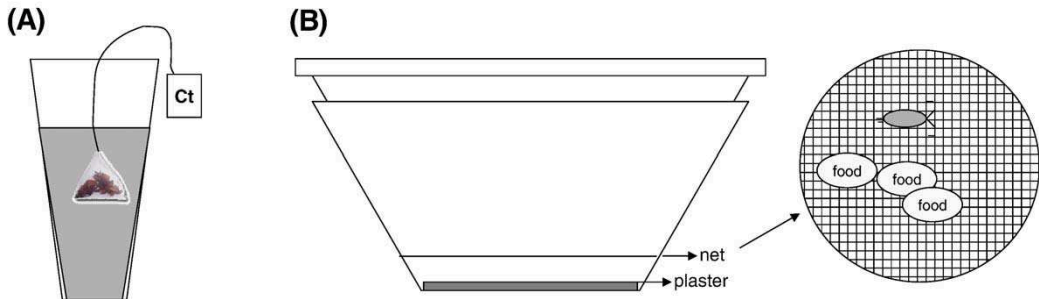

Fig. 14 Scheme for leaf contamination (A) and experimental test boxes (B) (Loureiro et al. 2006)

2.3 Leaf contamination

For the zinc sulphate exposure test, alder leaves were cut as disks (Ø 10 mm) and weighed (± 20 mg), enclosed in a net bag and submerged in the contaminated solution for 4 days. The concentrations of contaminant used were 20 and 100 mg/L to achieve around 5.5 and 9.5 µg Zn/g of leaf (Loureiro et al. 2006), and for the control, leaf disks were submerged in distilled water. For each concentration ten replicates were prepared. In the beginning of the experiments leaf disks were removed from the net bags, air dried and placed in the upper test boxes.

For the diazinon exposure test, a stock solution was made in ethanol and the concentration range in double-distilled water. The concentrations of contaminant used were 17.5 and 175 µg diazinon per gram dry food, and for the control, leafs disks were moistened with distilled water. Alder leaf disks were contaminated on the day of use and topically.

The final leaf concentration of 5.5 and 9.5 µg zinc / gram dry food has been based on the findings of (Loureiro et al. 2006). These values are the NOEC and LOEC values, respectively for the consumption ratio when exposed to the same conditions. The final leaf concentration of the pesticide diazinon (17.5 and 175 µg diazinon / gram dry food) has also been considered by (Vink et al. 1995) as NOEC and LOEC respectively on the energy budget of *P. pruinosus* when exposed to contaminated food.

2.4 Energy Reserves: Protein and Carbohydrate quantification

To determine total protein and carbohydrate content, isopods were sonicated with a sonicator in 600 µl distilled water after which 200 µl of 15% trichloroacetic acid (TCA)

was added and incubated at –20 ºC for 10 min. After centrifugation (1 000g, 10 min, 4ºC), the supernatant was separated has the carbohydrate fraction. The remaining pellet was resuspended in 2.5ml NaOH, incubated at 60 ºC for 30 min, after which it was neutralised with 1.5 ml HCl and used has the protein fraction.

Total protein content was then determined using Bradford's reagent (Bradford 1976), by measuring the absorbance at 590 nm using bovine serum albumin as a standard.

Total carbohydrate content was determined by adding 50 µl of 5% phenol and 200 µl H_2SO_4 to 50 µl of sample in a multiwell microplate, incubated for 30min at 20 ºC, and the absorbance was measured at 492 nm using glucose as a standard. The protein and carbohydrate content is expressed as mg/ mg org and J/mg org (expressed as fresh weight).

2.5 Energy Reserves: Lipid quantification

Total lipid quantification was based in the method described by (Bligh & Dyer (1959). Isopods were sonicated in 200 µl double-distilled water after which 500 µl chloroform (spectrofotometric grade) were added. After vortexed more 500 µl methanol (spectrofotometric grade) and 250 µl double-distilled water were added to the previous content, centrifuged (1 000g, 5min, 4ºC) and the top phase removed; the remaing phase was used for lipid measurement. To 100 µl of lipid extract were added 500 µl H_2SO_4 and heated for 15 min (200ºC); after cooling down, 1.5 ml of double-distilled water was added and total lipid content was determined by measuring the absorbance at 370 nm using tripalmitin as a standard. The lipid content is expressed as mg/ mg org and J/mg org (expressed as fresh weight).

2.6 Chemical compounds

All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany) and were all of high quality and purity.

2.7 Statistics

One-way analysis of variance (ANOVA) using the SigmaStat statistical package (SPSS 1999) was used to test for statistical differences between concentration treatments. Whenever significant differences were found a Dunnett's comparison test was

performed. Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal Wallis ANOVA on Ranks was performed, followed by the Dunnett's or Dunn's method when significant differences were found.

3 Results

3.1 Zinc sulphate exposure

The results obtained for *P. pruinosus* exposed to zinc sulphate are shown in Fig. 15. For carbohydrates it was observed a significant decrease for both 7-day and 14-day of exposure (Dunnett's test $p<0.001$). For lipids and proteins no significant differences where found.

For ETS a significant difference was found for 9.5 µg/g dry leaf at a 14-day exposure period, showing a decrease of 43% (Dunnett's test $p<0.001$).

Both zinc concentrations showed a significant decrease on the CEA value in day 7 (Dunnett's test $p<0.001$), but after 14 days of exposure only the highest concentration showed a significant decrease in this endpoint. Eventhough the response pattern was similar in both exposure periods.

A Two-Way ANOVA showed a significant interaction between concentrations and time for exposure only for ETS ($p=0.017$).

When we compare the control for time exposure no significant differences were found for all the parameters. The ETS controls values can not be compared since they represent two different time periods.

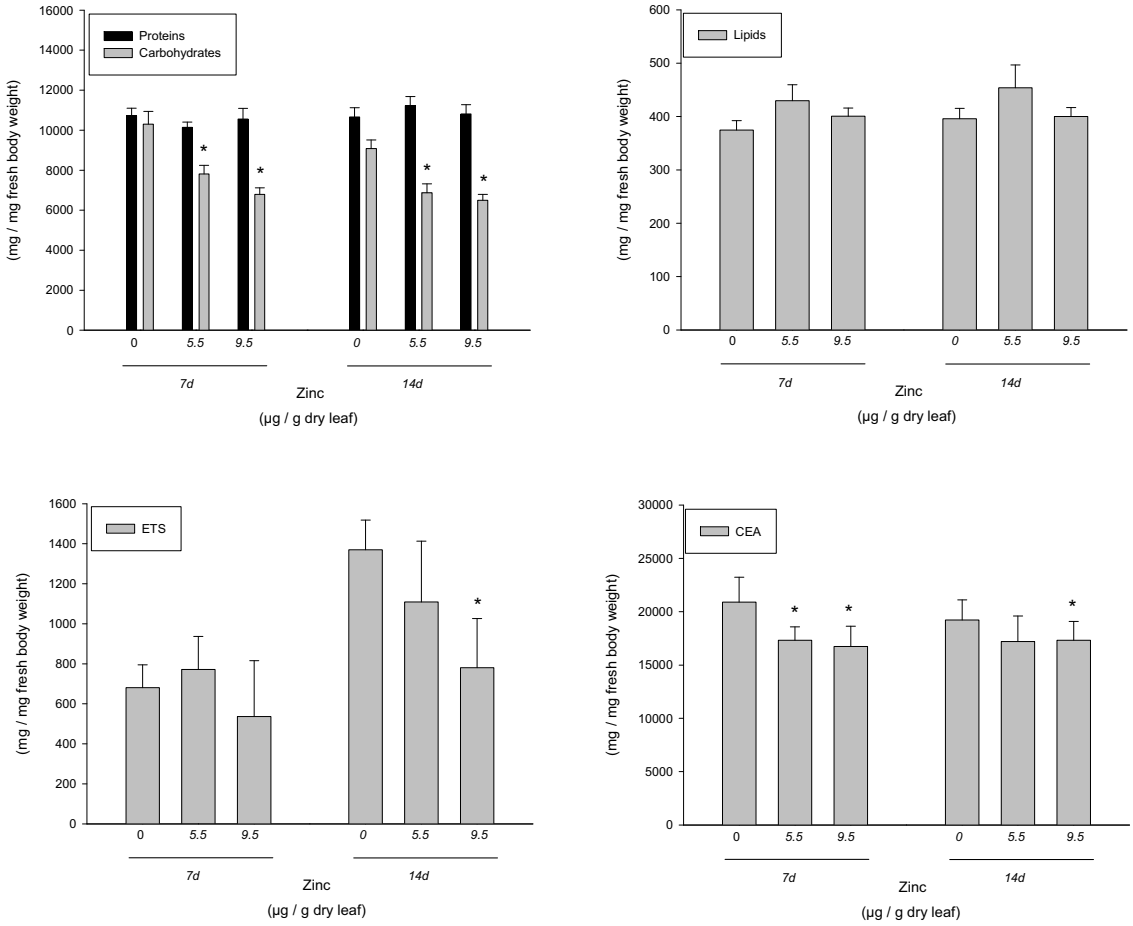

Fig. 15. The effects of Zinc sulphate on the cellular energy allocation parameters of *Porcellionides pruinosus*. Bars are mean values and corresponding standard error bars. CEA= Cellular Energy Allocation, ETS= Electron Transport System activity ***=** Dunnett's test, $p<0.05$

3.2 Diazinon exposure

The results obtained for *P. pruinosus* exposed to diazinon are shown in Fig. 16. It was observed a significant decrease on the carbohydrates content for both concentrations after 14 days of exposure (Dunnett's test $p<0.001$). For the lipids and proteins content no significant differences where found. Even though, protein and lipid contents increased when comparing the two exposure periods.

For the ETS no significant difference were observed on both exposure periods ($p>0.05$). For the CEA endpoint, it was observed a significant decrease for the highest concentration exposure but only after 14 days of exposure (Dunnett's test $p<0.001$).

A Two-Way ANOVA using as factors concentration and time exposure showed a significant interaction for carbohydrates (p<0.001) and CEA (p=0.005), which indicates that a long-time exposure will affect the organisms.

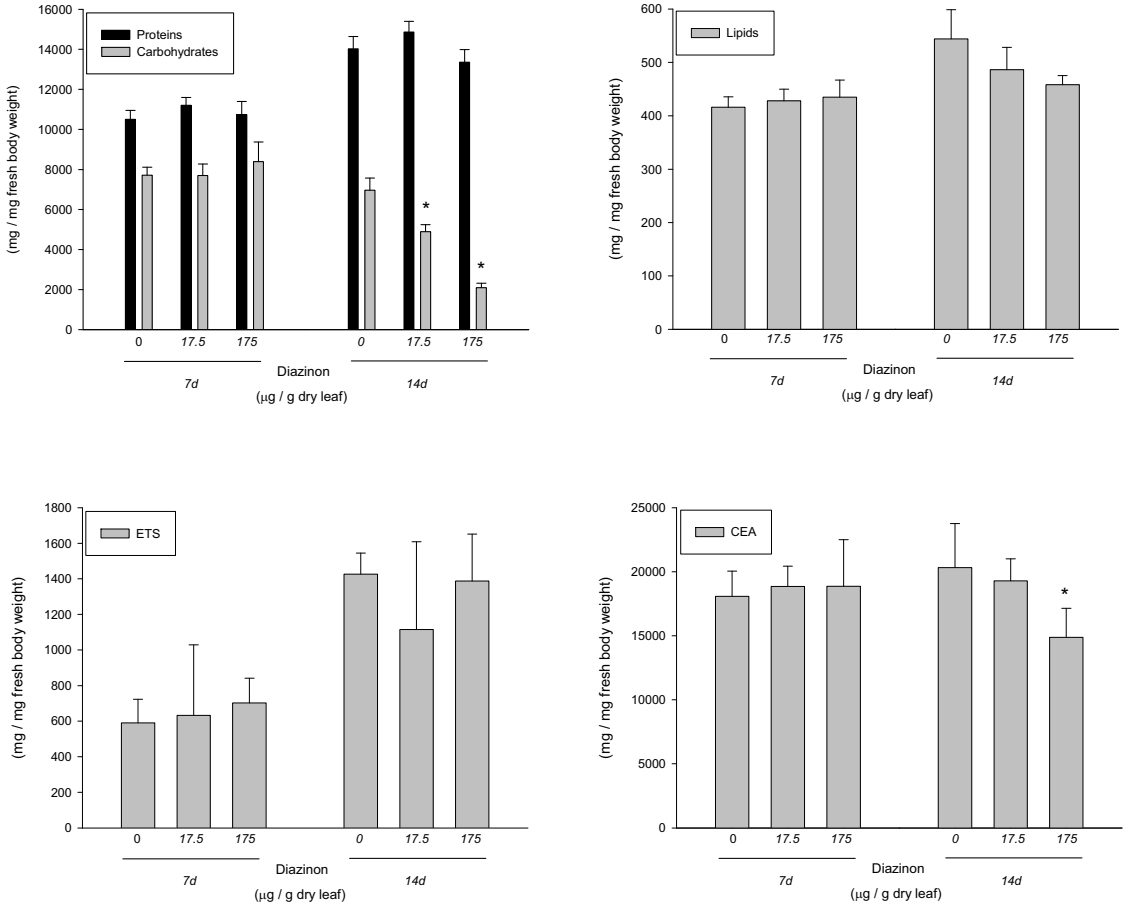

Fig. 16 The effects of diazinon on the cellular energy allocation parameters of *Porcellionides pruinosus*. Bars are mean values and corresponding standard error bars. CEA= Cellular Energy Allocation, ETS= Electron Transport System activity *= Dunnett's test, p<0.05

4 Discussion

The present work showed the energy budget of the isopod *Porcellionides pruinosus* exposed zinc sulphate and diazinon. Energy reserves are important for the maintenance, growth and reproduction requirements of all organisms. Energy reserves are normally stored as glycogen or lipids and are used whenever necessary, being normally consumed as a first step the carbohydrates, followed by lipids and then proteins.

This pattern was also observed in our study where differences were only found on the carbohydrates contents, showing that for these concentrations and time of exposure no other energy reserves needed to be used.

In certain cases and organisms, the primary source of energy can be the lipidc fraction instead of the carbohydrates, but only under severe stress conditions (i.e. xenobiotics) proteins are consumed, since normally they are produced for structural purposes.

For the zinc exposure the ETS was affected but not for diazinon exposure. The decrease in the ETS for the freshwater gastropods *Melanoides tuberculata* and *Helisoma duryi* when exposed to zinc was also observed by Moolman et al (2007), but no data was found to compare the non observed effect for the diazinon exposure.

As an overall result it was expected that the allocation of energy by cells was decreased as carbohydrates were used by the isopods, as energy source. This was observed after the 14 day of exposure and for the highest concentration of both chemical, although slight differences in response patterns were found in day 7 for both chemicals.
The use of ETS and CEA can give us a more accurate and understandable notion of the organisms' energy budget, because it will integrate the reserves measured and not use just one as an endpoint.

In the exposure to zinc sulphate significant decreases in carbohydrates is observe for both concentrations at a 7-day exposure period and continue to decrease in the 14-day exposure period, although there was no interaction between concentration and exposure time. The lipids show no differences between exposure time and concentrations, although it seems the 5.5 µg diazinon/g dry leaf concentrations lead to an increase for both the exposure periods. The proteins have the same pattern has lipids, also showing a stimulation in the 5.5 µg diazinon/g dry leaf concentrations.

When comparing our results with the ones obtained for Loureiro et al. (2006) where the same isopod *Porcellionides pruinosus* was exposed for a 14-day period to zinc sulphate, a significative decrease in the CEA for the higher contaminant concentration should be expected since at this concentration a significative decrease in the consumption rate was

observed. As for the 5.5 µg diazinon/g dry leaf concentration no significant changes were expected collaborating our results.

Organisms exposed to the pesticide diazinon showed no differences in carbohydrates for the 7-day exposure period, although a clear pattern of consumption of this energy reserve can be seem at 14-day period with significant differences in both concentrations. For lipids the same pattern for both periods is observed although the amount of lipids seems to be higher than the one found for 7-day period. When this data is compared with the work from Vink et al. (1995) also for the specie *Porcellionides pruinosus* exposed to diazinon, similar results are found for carbohydrates, but not for lipids were significant differences could be observed at lower concentrations (8.71 µg diazinon/g dry leaf).

The proteins show no differences for both periods, although such has in lipids the amount found for 14-day exposure period is higher them the one for 7-day exposure period. Although Vink et al. (1995) found no significant differences for lipids at even higher concentrations, the higher content of lipids and proteins for the 14-day exposure period can not been explained since carbohydrate content for 1-day exposure continue to decrease from the ones observed at the 7-day exposure period.

The CEA shows that although no differences where found at a 7-day exposure period, at a 14-day exposure period a decrease patter can be notice, which means that organisms can became seriously affected, results also observed by Moolman et al (2007), for the freshwater gastropods *Melanoides tuberculata* and *Helisoma duryi* when exposed to zinc where a 75% in the CEA is observed for both species.

The use of ETS and CEA along with energy reserves content provided a sensitive measure of the decreased energy budget resulting from sublethal metal and can be used as biomarkers. However, the of supplementary biomarkers may contribute to better understand the mechanisms of these specific responses of organisms.

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Chapter V

Discussion and Conclusion

Discussion and conclusion

Harmful compounds like the heavy metal zinc or the pesticide diazinon can affect organisms, increasing their mortality rates, interfering with their feeding processes, energy storage or predator avoidance and can lead to a slow population growth, low reproduction and ultimately to population decline or even extinction.

To avoid or diminish these effects, organism can use their own detoxification mechanisms, increasing detoxification enzymes, wasting energy reserves, or modifying life strategies.

In a general analyse on the effects of zinc in *Porcellionides pruinosus* the results suggest that this metal causes oxidative stress to the organism, with clear patterns of inhibition on the metal-binding enzymes and an increase in lipid peroxidation (LPO). This inhibition pattern shows that the phase II detoxification is not working properly. The lack of the oxidative stress biomarkers activity can also be connected to a decrease in the carbohydrate content and a decrease in the organism normal activity and leading to a decrease on energy consumption (ETS) and possibly being an easy target for predators.

For the effects of diazinon to this terrestrial isopod this work suggests that almost all the oxidative stress biomarkers have no changes on their activity and that the biomarker that shows more variation is AChE, which is know to be this chemical target enzyme. A decrease in the AChE activity was expected and would lead to organisms' paralysation which should be supported by a lower cellular energy allocation index (CEA) caused by a decrease in carbohydrates and lipids. The significant carbohydrate decrease should also be explained by the crescent glucose need in the nervous central system due to the AChE inhibition. Although the carbohydrate and CEA index supports the inhibition of the AChE activity, the results obtained in this work come in contradiction.

This work will be useful for further investigations, since a base has been establish for the use of biomarkers activity and energy reserves content on isopods for environmental risk assessment purposes. For example the mean values obtained in this work for the

biomarkers and energy reserves were almost all within basal level range. The basal levels quantification of the biomarkers activity and energy reserves content, along with the acetylcholinesterase characterization will be useful for further comparisons along organisms taken from different contaminated sites. In the same way, future works should be based on the analyses of patterns in biomarkers and energy reserves resulting for other contaminants families and the response to abiotic factors to also serve as reference.